PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of:

LIAT MINTZ

CASE NO: 28238

SERIAL NO: 10/659,782

GROUP ART UNIT: 1636

FILED: SEPTEMBER 11, 2003

EXAMINER: J. DUNSTON

FOR: COMPOSITIONS, REAGENTS AND KITS FOR AND METHODS OF DIAGNOSING, MONITORING AND TREATING OBESITY AND/OR DIABETES

DECLARATION UNDER 37 C.F.R. § 1.132

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Sir:

I, Liat Mintz, declare that:

I am a citizen of Israel and reside at 2 Kulessa Court, East Brunswick, New Jersey 08816.

I am the founder of DiaLean Ltd., an Israeli corporation.

I received a Ph.D. in Immunology and Cell Research from the Tel-Aviv University, Tel-Aviv, Israel; an M.Sc. in Microbiology from the Tel-Aviv University, Tel-Aviv, Israel; and a B.Sc. in Life Sciences from the Tel-Aviv University, Tel-Aviv, Israel.

I am the Applicant of the above-identified application.

The following are my remarks:

1. The Office Action indicated that claims 31-34 and 47-48 were rejected under 35 U.S.C. § 101 because the claimed invention is not supported by either a specific and substantial asserted utility or a well-established utility.

2. The Examiner asserted that "it is not clear whether the Ghrelin Variant 2 protein [SEQ ID NO:32] is intended to act as agonist or antagonist of physiological pathways related to diabetes and/or obesity. Further, there is no art

of record that discloses the function of the Ghrelin Variant 2 protein. Therefore, the asserted utility of the treatment of diabetes and/or obesity with the Ghrelin Variant 2 protein is not supported by a well established utility."

3. The Examiner further stated that "the asserted utility of treatment of obesity and/or diabetes is not substantial because the function and effects of the protein are not known. Further experimentation would be required to determine a 'real world' context for the Ghrelin Variant 2 protein in the treatment of diabetes and/or obesity."

4. Attached hereto are a series of experiments which establish a specific and substantial asserted utility for the claimed inventions. The experiments are essentially the same as those disclosed in Tschöp M. *et al.*, Ghrelin induces adiposity in rodents, Nature 407:908-13 (2000), a copy of which is attached hereto.

5. The basic experimental procedure is as follows. Fifty adult male 129S2/SvHsd mice ("129Sv mice") were assigned to five test groups after a pre-test conditioning period. Test compounds used in the experiments were human acylated SEQ ID NO:32 ghrelin and human des-acyl SEQ ID NO:32 ghrelin with human acylated ghrelin and human des-acyl ghrelin as controls (Test Groups 1M, 2M, 3M, and 4M). Vehicle (1.6% mannitol) was used a general control for the experimental system (Test Group 5M).

6. Acylated ghrelin is so-named because of the presence of an octanoyl group on the hydroxy group of serine in amino acid position 3 of the ghrelin protein. In the organism, this peptide modification is unique. It is a post-translational modification, which is most likely essential for the function of this peptide in the organism (Bednarek M.A. *et al.*, Structure-function studies on the new growth hormone-releasing peptide, ghrelin: minimal sequence of ghrelin necessary for activation of growth hormone secretagogue receptor 1a, J. Med. Chem. 43:4370-76 (2000), a copy of which is attached hereto). Des-acyl ghrelin, while present *in vivo*, is thought to have to no significant endocrine activity (Asakawa A. *et al.*, Stomach regulates energy balance via acylated ghrelin and desacyl ghrelin, Gut 54:18-24 (2005), a copy of which is attached hereto).

7. The experiments were designed to test the effectiveness of not only the acylated form of SEQ ID NO:32, but also the des-acyl form of the SEQ ID NO:32 splice variant.

8. Test and Control compounds were administered in a blinded fashion once daily over five successive days (Days 0-4). Administration was effectuated by subcutaneous injection in a rotating fashion between the left and right sides of the dorsal trunk of the mice on successive days.

9. The only significant difference in experimental procedure between the present experiments and those disclosed in Tschöp *et al.* is that, here, only 20 µg of the respective test compounds were administered to the mice, compared to 200 µg in Tschöp *et al.* Usage of lower dosage amounts herein was chosen based on lower dosage amounts published in later experiments by several groups: Wren A. M., *et al.*, The novel hypothalamic peptide ghrelin stimulates food intake and growth hormone secretion, Endocrinology 141:4325-28 (2000), a copy of which is attached hereto; Asakawa A *et al.*, Antagonism of ghrelin receptor reduces food intake and body weight gain in mice, Gut 52:947-52 (2003), a copy of which is attached hereto; and Sun Y. *et al.*, Ghrelin stimulation of growth hormone release and appetite is mediated through the growth hormone secretagogue receptor, Proc. Natl. Acad. Sci. USA 101:4679-84 (2004), a copy of which is attached hereto.

10. Route of administration, frequency of administration, and duration of administration were all performed as described in Tschöp *et al.*

11. Determination of individual body weights of animals was made once daily during observation period in the morning, at about the same hour of the day. Body weight measurements were performed serially as per animal number, and not as per group.

12. Daily measurements of food consumption were made approximately for a 24-hour period. Determinations of food consumption are based on subtracting unused diet from the provided diet in hoppers. Food consumption measurements were performed serially as per animal number, and not as per group.

13. Evaluation of the potential effect of human acylated SEQ ID NO:32 ghrelin, human des-acyl SEQ ID NO:32 ghrelin, wild type human acylated ghrelin, and wild type human des-acyl ghrelin was primarily based on the relative and comparable measurement of body weight and food intake, expressed as mean group values, of the Test Compound-treated group versus the vehicle control group. Statistical analysis was performed using two-tailed Student's t-Test.

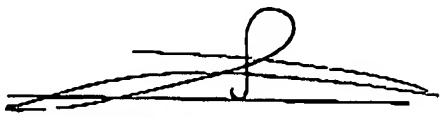
14. The cumulative body weight gain of the SEQ ID NO:32-treated and the wild type ghrelin-treated 129Sv mice was significantly higher ($p = 0.011$, $p = 0.010$ respectively) than the vehicle-treated controls. The cumulative body weight gain of the des-acyl SEQ ID NO:32-treated 129Sv mice was significantly lower ($p = 0.007$) than the vehicle-treated controls. In contrast, no significant differences ($p > 0.05$) were observed in the cumulative body weight gain between wild type des-acyl ghrelin-treated and vehicle-treated 129Sv mice.

15. The cumulative food consumption of the SEQ ID NO:32-treated and the wild type ghrelin treated 129Sv mice was significantly higher ($p = 0.024$, $p = 0.025$ respectively) than the vehicle-treated controls. The cumulative food consumption of the des-acyl SEQ ID NO:32-treated 129Sv mice was significantly lower ($p = 0.0079$) than the vehicle-treated controls. In contrast, no significant differences ($p > 0.05$) were observed in the cumulative food consumption between wild type des-acyl ghrelin-treated and vehicle-treated 129Sv mice.

16. Thus, acylated SEQ ID NO:32, like wild type ghrelin, significantly promotes body weight gain and food consumption. Des-acyl SEQ ID NO:32 significantly promotes weight loss and decreased food consumption, unlike wild type des-acyl ghrelin which has no significant influence on body weight and food consumption.

17. I believe that the experiments conducted thus establish a specific and substantial utility for the claimed inventions.

18. I declare that all statements made herein are either based on my own knowledge and are true, or if based on information and belief are believed to be true. I also declare that all statements were made with knowledge that willful false statements, and the like, are punishable by either fine, or imprisonment, or both under Section 1001 of Title 18 of the United States Code, and any such willful false statements may jeopardize the validity of either the patent application, or any patent issuing thereon.

By: 

Liat Mintz, Ph.D.

Dated: 10/25/05

Effect on Body Weight and Food Intake following Daily Subcutaneous Administration in 129Sv Male Mice of:

Human acylated ghrelin (Lot No. 3000212)
Human Des-acyl Ghrelin (Lot No. 3000182)
Human Acylated SEQ ID 32 (Lot No. 3000394)
Human Des-acyl SEQ ID 32 (Lot No. 3000418)

1. SUMMARY 2
2. OBJECTIVE 3
3. GUIDELINE 3
4. TEST MATERIALS 3
5. TEST SYSTEM 6
6. CONSTITUTION OF TEST GROUPS AND DOSE LEVELS 7
7. TEST PROCEDURES 8
8. EXAMINATIONS AND OBSERVATIONS 9
9. DATA EVALUATION 9
10. ANIMAL CARE AND USE STATEMENT 9
11. RESULTS 10
12. CONCLUSION 11

1. SUMMARY

1.1 The currently reported study was carried out to test the potential effect of Test Items ***Human Acylated Ghrelin*** (Ghrelin), ***Human Des-acyl Ghrelin, Human Acylated SEQID32 and Human Des-acyl SEQID32*** on body weight and food intake in 129Sv male mice.

1.2 The Test Items (20μg) or the Vehicle (1.6% mannitol) were administered once daily for 5 successive days, via the subcutaneous (SC) route, to groups comprising *n=10* 129Sv male mice.

1.3 No mortality occurred in any of the animals throughout the entire study period.

1.4 No clinical signs were observed in any of the animals throughout the entire study period.

1.5 The cumulative body weight gain, of the SEQID32-treated and Ghrelin-treated 129Sv mice was significantly higher ($p = 0.011$, $p = 0.010$ respectively) than the Vehicle-treated controls (1.04g, 1.6g and 0.38g, respectively). There were no significant differences between the groups during the *Conditioning Period* preceding the treatment period ($p > 0.05$). The cumulative body weight gain, of the Des-acyl SEQID32-treated 129Sv mice was significantly lower ($p = 0.007$) than the Vehicle-treated controls (0.12g and 0.38g, respectively). There were no significant differences between the groups during the *Conditioning Period* preceding the treatment period ($p > 0.05$). In contrast, no significant differences ($p > 0.05$) were observed in the cumulative body weight gain between the Des-acyl ghrelin-treated and Vehicle-treated 129Sv mice (0.37g and 0.38g, respectively).

1.6 The cumulative food consumption of the SEQID32-treated and Ghrelin-treated 129Sv mice was significantly higher ($p = 0.024$ and $p = 0.025$ respectively) than the Vehicle-treated controls (20.82g 21.73g, and 18.47g, respectively). There were no significant differences between the groups during the *Conditioning Period* preceding the treatment period ($p > 0.05$). The cumulative food consumption of the Des-acyl SEQID32-treated 129Sv mice was significantly lower ($p = 0.0079$) than the Vehicle-treated controls (17.56g and 18.47g, respectively). There were no significant differences between the groups during the *Conditioning Period* preceding the treatment period ($p > 0.05$). In contrast, no significant differences ($p > 0.05$) were observed in the cumulative food consumption between the Des-acyl ghrelin-treated and Vehicle-treated 129Sv mice (19.0g and 18.47g, respectively).

1.7 Under the prevailing experimental conditions of this study, and in view of the findings obtained, it may be concluded that ***Human Acylated SEQID32 (Lot No.***

3000394) and ***Human Acylated ghrelin (Lot No. 3000212)*** significantly promote body weight gain and food consumption in 129Sv, male mice. ***Human Des-acyl SEQID32 (Lot No. 3000418)*** significantly promotes weight loss and decreased food consumption while ***Human Des-acyl ghrelin (Lot No. 3000418)*** has no significant influence on boy weight and food consumption.

2. OBJECTIVE

Assessing the effect of the Test Items ***Human Acylated Ghrelin*** (Ghrelin), ***Human Des-acyl Ghrelin, Human Acylated SEQID32 and Human Des-acyl SEQID32*** on body weight and food intake, following daily subcutaneous (SC) administration to 129Sv mice.

3. GUIDELINE

This study design was essentially based on a published document (Tschop *et al.* 2000. Ghrelin induces adiposity in rodents. Nature. 407: 908-913).

4. TEST MATERIALS

4.1 Test Items:

(i) Name: Human acylated Ghrelin
Lot No.: 3000212
Supplied by: Dialean Ltd.
Manufacturer: BACHEM
Physical State: White Powder
Storage Conditions: -18 to -22°C until preparation

(ii) Name: Human Des-acylated Ghrelin
Lot No.: 3000182
Supplied by: Dialean Ltd.
Manufacturer: BACHEM
Physical State: White Powder
Storage Conditions: -18 to -22°C until preparation

(iii) Name: Human acylated SEQID 32
Lot No.: 3000394
Supplied by: Dialean Ltd.
Manufacturer: BACHEM
Physical State: White Powder

Storage Conditions: -18 to -22°C until preparation

(iv) Name: Human Des-acyl SEQID 32
Lot No.: 3000418
Supplied by: Dialean Ltd.
Manufacturer: BACHEM
Storage Conditions: -18 to -22°C until preparation

4.2 Control Item (vehicle) Components:

Name: D-Mannitol
Lot No.: 014K01101
Supplied by: Testing Facility
Manufacturer: Sigma-Aldrich
Physical State: Powder
Storage Conditions: 18 - 28°C
Expiry Date: September 2010

Name: Water for Injection
Lot No.: 4072-51
Supplied by: Testing Facility
Manufacturer: Norbrook Laboratories
Storage Conditions: 2-8°C
Expiry Date: February 2006

4.3 Adjunct Item:

Name: 0.9% Sodium Chloride (Physiological Saline)
Batch No.: WP 5B08 5
Characteristics: Clear liquid
Supplied by: Testing Facility
Manufacturer: Teva Medical
Storage Conditions: 2-8°C
Expiry Date: February 2007

4.4 Preparation of Test Materials:

4.4.1 4% Mannitol Solution: Prepared by dissolving D-Mannnitol in *Water for Injection* to achieve final concentration of 40 mg/ml.

4.4.2 Test Items *Stock Solution*: Each Test Item (5mg) is dissolved in 10 ml of 4% Mannitol solution, divided into aliquots and kept frozen (-70°C) until the time of use.

4.4.3 Test Items *Dosing Solutions:* On each day of dosing, the required amount of each Test Item is thawed and diluted with Physiological Saline to a concentration of 0.2 mg/ml.

4.4.4 Control Item *Dosing Solution*: Prepared on each day of dosing, by diluting 4% Mannitol Solution with Physiological Saline at the same ratio as the Test Items *Dosing Solutions*.

All preparation procedures were performed under sterile conditions. Test and Control Item solutions are kept on ice and equilibrated to room temperature shortly prior to dosing (time is recorded).

5. TEST SYSTEM

5.1	**Species/Strain:**	Mouse / 129S2/SvHsd
5.2	**Source:**	129Sv – Harlan UK, Ltd. (ISO 9001:2000 Certificate No.: US2002/3081)
5.3	**Gender:**	Male
5.4	**Total No. of Animals:**	*n = 50*
5.5	**Age:**	Healthy adult, 10-12 weeks of age at study initiation.
5.6	**Acclimation:**	At least 5 days.
5.7	**Animals Health:**	The health status of the animals used in this study is examined on arrival. Only animals in good health are acclimatized to laboratory conditions and are used in the study.
5.8	**Housing:**	During acclimation and throughout the study period, the animals are housed within a limited access Specific Pathogen Free (SPF) rodent facility and kept individually in polypropylene cages (23 × 17 × 14 cm), fitted with solid bottoms and filled with wood shavings as bedding material (7083 Harlan Teklad Shredded Aspen). Laboratory analysis of the finished bedding product is performed quarterly, and provides information on potential contaminants such as pesticides, heavy metals, yeast and molds. The most relevant QC test result/s is/are included in the archives with the study data.
5.9	**Food and Water:**	Animals are provided *ad libitum* a commercial rodent diet (Harlan Teklad *2018S* Global 18% Protein Rodent Diet), and free access to drinking water, supplied to each cage via polyethylene bottles with stainless steel sipper tubes. Feedlot analysis of the diet batch used in the study is included in the archives with the study data. The water is filtered (0.1µ filter), chlorinated and acidified. The water is monitored for contaminants twice yearly.
5.10	**Environment:**	Automatically controlled environmental conditions are set to maintain temperature at

24 – 26°C with a relative humidity (RH) of 30-70%, a 12-hr light/12-hr dark cycle and 15-30 air changes/hr in the study room. Temperature and RH are monitored daily. The light cycle is monitored by the control computer.

5.11 Identification: Animals are given a unique animal identification ear number. This number appears on a cage card, visible on the front of each cage. The cage card additionally contains the study number and relevant details as to treatment group and dose level.

5.12 Randomization: At the end of the *Pre-Test Conditioning* period, animal were assigned to groups in a way that the mean body weight ±SD are similar as possible among all groups.

5.13 Termination: At the end of the study surviving animals are euthanized by CO_2 asphyxiation and subjected to necropsy.

5.14 Justification: The 129Sv strain is selected based on previously published literature (Tschop *et al.*) and according to the preceding HBI Study Report DLN/002/EM.

6. CONSTITUTION OF TEST GROUPS AND DOSE LEVELS

Group No.	Strain	Individual Animal No.	Treatment	Route	Dose
1M	129Sv	*5,6,10,16,40,46,47,54,57,71*	Acylated SEQID 32	Daily (6 days) SC injection – dorsal trunk	20µg / 0.1 ml / animal / day
2M		*1,3,7,31,32,38,41,45,48,68*	Des-acyl SEQID 32		20µg / 0.1 ml / animal / day
3M		*9,14,15,18,23,37,39,44,63,76*	Des-acyl ghrelin		20µg / 0.1 ml / animal / day
4M		*2,24,25,35,36,43,50,51,61,74*	Acylated ghrelin		20µg / 0.1 ml / animal / day
5M		*26,27,30,33,49,56,58,60,64,69*	Vehicle		0.1 ml / animal / day

7. TEST PROCEDURES

7.1 Principles of the Test:

Test and Control Items were administered once daily during 5 successive days (Days 0-4). Test Items *Dosing Solutions* are administered in a blinded fashion where their identity is unknown to the animal technicians or the Study Director. Once animals are assigned to the different groups, the order of administration per group is performed in a rotating order, as depicted for example in the table below, to avoid any accumulative effect of the time gap between dosing time and the dark hour's period:

	Day No.					
Order	**1**	**2**	**3**	**4**	**5**	**6**
1st	1M	2M	3M	4M	5M	1M
2nd	2M	3M	4M	5M	1M	2M
3rd	3M	4M	5M	1M	2M	3M
4th	4M	5M	1M	2M	3M	4M
5th	5M	1M	2M	3M	4M	5M

7.2 *Pre-Test Conditioning* of Animals:

At the 4 preceding days to initiation of Test and Control Items administration (Study Days -4 to -1), all animals were subjected to daily SC administration of Physiological Saline, as well as body weight and food consumption measurements, in attempt to diminish the procedural stress-related effects on the measured parameters.

7.3 Administration:

The Test and Control Items were administered by SC injection in a rotating fashion between the left and right sides of the dorsal trunk on successive days. Each dosing session is carried out at the last 1½ hours of the light period.

7.4 Dose Levels:

Animals of the Test Items groups were administered with 20μg of the respective Test Item per day. In all cases, the dose volume is 0.1 ml/animal/day.

7.5 Justification for Route of Administration, Dose, Frequency and Duration:

The route of administration, frequency and duration are selected based on previously published literature (Tschop *et al.*). The dose level is lower than that used by Tschop.

8. EXAMINATIONS AND OBSERVATIONS

8.1 Observation Period:

Animals were observed for 10 successive days, starting with 4-day *Pre-Test Conditioning* period and continued with 5 -day Treatment Period.

8.2 Body Weight:

Determination of individual body weights of animals was made once daily during observation period in the morning, at about the same hour of the day. Body weight measurements were performed serially as per animal number, and NOT as per group.

8.3 Food consumption:

Daily measurements of food consumption were made for approximately 24-hour period. Determinations of food consumption are based on subtracting unused diet from the provided diet in hoppers. Food consumption measurements were performed serially as per animal number, and NOT as per group.

8.4 Clinical Signs:

Detailed Clinical Signs were recorded once daily throughout the study period. Observations included changes in skin, fur, eyes, mucous membranes, occurrence of secretions and excretions (e.g. diarrhea) and autonomic activity (e.g. lacrimation, salivation, piloerection, pupil size, unusual respiratory pattern) changes in gait, posture and response to handling, as well as the presence of bizarre behavior, tremors, convulsions, sleep and coma.

9. DATA EVALUATION

Evaluation of the potential effect of ***Human acylated SEQID 32, Human Des-acyl SEQID 32, Human Acylated Ghrelin*** and ***Human Des-acyl ghrelin*** was primarily based on the relative and comparable measurement of body weight and food intake, expressed as mean group values, of the Test Item treated group *vs*. the vehicle control group. Statistical analysis was performed using two-tailed Student's t-Test.

10. ANIMAL CARE AND USE STATEMENT

This study is performed following the review by the Committee for Ethical Conduct in the Care and Use of Laboratory Animals of the Hebrew University, Jerusalem, the IACUC responsible for approving HBI animal usage application (Approval No. MD

106.27-2) and in compliance with its respective registration under: NIH accreditation No.: OPRR-A01-5011 HU.

11. RESULTS

11.1 **Mortality** (Table 1):

No mortality occurred in any of the animals throughout the entire study period.

11.2 **Clinical Signs** (Table 2):

No clinical signs were observed in any of the animals throughout the entire study period.

11.3 **Body Weight** (Tables 3-5 & I, Figures 1-3):

The cumulative body weight gain, of the SEQID32-treated and Ghrelin-treated 129Sv mice was significantly higher ($p = 0.011$, $p = 0.010$ respectively) than the Vehicle-treated controls (1.04g, 1.8g and 0.38g, respectively). There were no significant differences between the groups during the *Conditioning Period* preceding the treatment period ($p > 0.05$). The cumulative body weight gain, of the Des-acyl SEQID32-treated 129Sv mice was significantly lower ($p = 0.007$) than the Vehicle-treated controls (0.12g and 0.38g, respectively). There were no significant differences between the groups during the *Conditioning Period* preceding the treatment period ($p > 0.05$). In contrast, no significant differences ($p > 0.05$) were observed in the cumulative body weight gain between the Des-acyl ghrelin-treated and Vehicle-treated 129Sv mice (0.37g and 0.38g, respectively).

11.4 **Food Consumption** (Tables 6, 7 & II, Figures 4 & 5):

The cumulative food consumption of the SEQID32-treated and Ghrelin treated 129Sv mice was significantly higher ($p = 0.024$ and $p = 0.025$ respectively) than the Vehicle-treated controls (20.82g 21.73g, and 18.47g, respectively). There were no significant differences between the groups during the *Conditioning Period* preceding the treatment period ($p > 0.05$). The cumulative food consumption of the Des-acyl SEQID32-treated 129Sv mice was significantly lower ($p = 0.0079$) than the Vehicle-treated controls (17.56g and 18.47g, respectively). There were no significant differences between the groups during the *Conditioning Period* preceding the treatment period ($p > 0.05$). In contrast, no significant differences ($p > 0.05$) were observed in the cumulative food consumption between the Des-acyl ghrelin-treated and Vehicle-treated 129Sv mice (19.0g and 18.47g, respectively).

12. CONCLUSION

Under the prevailing experimental conditions of this study, and in view of the findings obtained, it may be concluded that ***Human Acylated SEQID32 (Batch No. 3000394)*** and ***Human Acylated ghrelin (Lot No. 3000212)*** significantly promote body weight gain and food consumption in 129Sv, male mice. ***Human Des-acyl SEQID32 (Lot No. 3000418)*** significantly promotes weight loss and decreased food consumption while ***Human Des-acyl ghrelin (Lot No. 3000182)*** has no significant influence on boy weight and food consumption.

Table 1: Mortality Incidence Observed in 129Sv Mice throughout the 6-day Repeated (1× daily) SC Administration of *Human acylated ghrelin (Lot No. 3000212), Human Des-acyl Ghrelin (Lot No. 3000182), Human Acylated SEQ ID 32 (Lot No. 3000394), Human Des-acyl SEQ ID 32 (Lot No. 3000418):*

Group No. & Sex	Group Size	Strain	Treatment	MORTALITY (Number affected per total number of animals)
1M	*n=10*	129Sv	Acylated SEQID 32	0/10
2M	*n=10*		Des-acyl SEQID 32	0/10
3M	*n=10*		Des-acyl ghrelin	0/10
4M	*n=10*		Acylated ghrelin	0/10
5M	*n=10*		Vehicle	0/10

Table 2: Clinical Signs observed in 129Sv Mice throughout the Entire 9-day Observation Period Including the 6-day Repeated (1× daily) SC Administration of *Human acylated ghrelin (Lot No. 3000212), Human Des-acyl Ghrelin (Lot No. 3000182), Human Acylated SEQ ID 32 (Lot No. 3000394), Human Des-acyl SEQ ID 32 (Lot No. 3000418):*

Group No. & Sex	Group Size	Strain	Treatment	OBSERVATION	
				Pre-Test Conditioning Period (Days -4 to -1)	Days 0-4
1M	*n=10*	129Sv	Acylated SEQID 32	NAD	NAD
2M	*n=10*		Des-acyl SEQID 32	NAD	NAD
3M	*n=10*		Des-acyl ghrelin	NAD	NAD
4M	*n=10*		Acylated ghrelin	NAD	NAD
5M	*n=10*		Vehicle	NAD	NAD

NAD = No Abnormality Detected

Table 3: Mean (± SD) Group Body Weight Values (g) of 129Sv Mice Throughout the Entire 10-d Observation Period, Including the 4-day pre-conditioning period and the 5-day Treatment Period.

Group		Study Day No									
		Pre-Test Conditioning Period				Test Item Administration Period					
		-4	-3	-2	-1	0	1	2	3	4	5
1 Acylated SEQID 32	Mean	**25.8**	**26.4**	**26.6**	**26.8**	**27.1**	**27.4**	**27.5**	**27.8**	**27.9**	**28.1**
	±SD	1.8	1.6	1.7	1.6	1.7	1.8	1.8	2.0	2.0	1.9
	n=	*10*	*10*	*10*	*10*	*10*	*10*	*10*	*10*	*10*	*10*
2 Des-acyl SEQID 32	Mean	**26.5**	**26.9**	**27.0**	**27.0**	**27.1**	**27.0**	**26.8**	**27.1**	**27.2**	**27.2**
	±SD	1.6	1.6	1.6	1.8	1.5	1.5	1.7	1.4	1.5	1.5
	n=	*10*	*10*	*10*	*10*	*10*	*10*	*10*	*10*	*10*	*10*
3 Des-acyl Ghrelin	Mean	**26.3**	**26.7**	**27.0**	**26.8**	**27.1**	**27.1**	**27.0**	**27.3**	**27.5**	**27.5**
	±SD	1.4	1.5	1.7	1.7	1.6	1.4	1.5	1.6	1.6	1.7
	n=	*10*	*10*	*10*	*10*	*10*	*10*	*10*	*10*	*10*	*10*
4 Acylated Ghrelin	Mean	**26.2**	**26.5**	**26.7**	**26.9**	**27.1**	**27.6**	**27.9**	**28.3**	**28.5**	**28.7**
	±SD	1.5	1.5	1.6	1.7	1.6	1.7	1.6	1.7	1.7	1.9
	n=	*10*	*10*	*10*	*10*	*10*	*10*	*10*	*10*	*10*	*10*
5 Control	Mean	**26.1**	**26.8**	**26.9**	**26.8**	**27.0**	**27.2**	**27.0**	**27.3**	**27.3**	**27.4**
	±SD	1.3	1.3	1.4	1.5	1.6	1.7	1.6	1.6	1.4	1.5
	n=	*10*	*10*	*10*	*10*	*10*	*10*	*10*	*10*	*10*	*10*

Figure 1: Mean Group Body Weight Values (g) of 129Sv Throughout the Entire 10-d Observation Period, Including the 4-day pre-conditioning period and the 5-day Treatment Period.

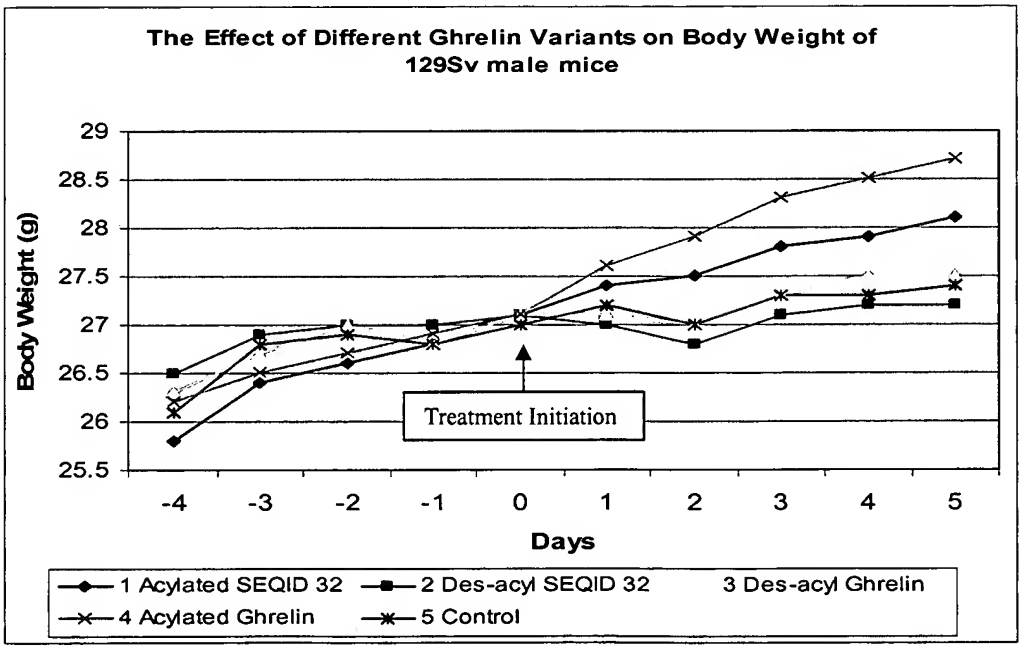


Table 4: Mean (± SD) Group Daily Body Weight (BW) Gain Values (g) of 129Sv Throughout the 5-day Treatment Period.

Group		Study Day No Treatment Period						
	Day	0	1	2	3	4	5	Daily BW Gain
1 Acylated SEQID 32	Mean	0	0.3	0.2	0.3	0.2	0.2	0.166
	±SD	0.0	0.37	0.28	0.50	0.27	0.32	
	n=	10	10	10	10	10	10	6
2 Des-acyl SEQID 32	Mean	0	0.0	-0.2	0.2	0.1	0.0	0.016
	±SD	0	0.30	0.42	0.42	0.33	0.28	
	n=	10	10	10	10	10	10	6
3 Des-acyl Ghrelin	Mean	0	0.0	-0.1	0.3	0.2	0.0	0.066
	±SD	0	0.50	0.28	0.30	0.19	0.28	
	n=	10	10	10	10	10	10	6
4 Acylated Ghrelin	Mean	0	0.6	0.2	0.4	0.3	0.2	0.283
	±SD	0	0.39	0.25	0.45	0.43	0.30	
	n=	10	10	10	10	10	10	6
5 Control	Mean	0	0.1	-0.2	0.3	0.1	0.1	0.066
	±SD	0	0.29	0.38	0.31	0.29	0.38	
	n=	10	10	10	10	10	10	6

Figure 2: Mean Group Daily Body Weight Gain Values (g) of 129Sv Mice Throughout the Entire 5-d Treatment Period.

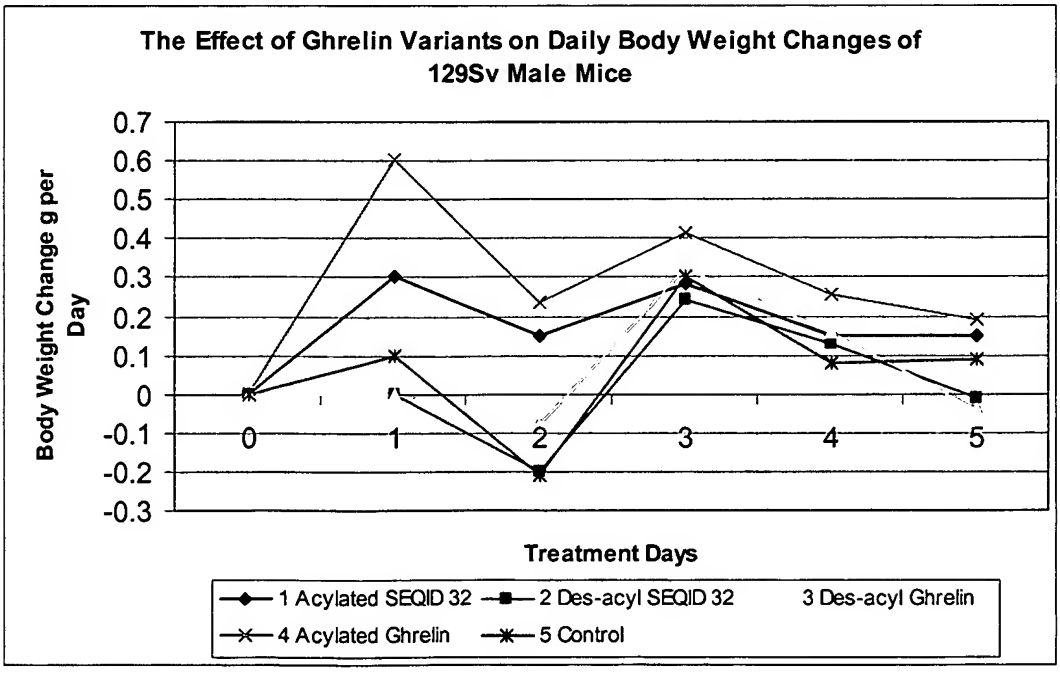


Table 5: Mean (± SD) Group Cumulative Body Weight (BW) Gain Values (g) of 129Sv Mice Throughout the 5-day Treatment Period.

Group		Study Day No Treatment Period					
	Day	0	0 to 1	0 to 2	0 to 3	0 to 4	0 to 5
1 Acylated SEQID 32	Mean	**0**	**0.31**	**0.46**	**0.74**	**0.89**	**1.04**
	±SD	0	0.36	0.40	0.44	0.43	0.38
	n=	*10*	*10*	*10*	*10*	*10*	*10*
2 Des-acyl SEQID 32	Mean	**0**	**-0.04**	**-0.24**	**0**	**0.13**	**0.12**
	±SD	0	0.29	0.31	0.33	0.34	0.33
	n=	*10*	*10*	*10*	*10*	*10*	*10*
3 Des-acyl Ghrelin	Mean	**0**	**0.01**	**-0.07**	**0.25**	**0.41**	**0.37**
	±SD	0	0.50	0.40	0.30	0.43	0.49
	n=	*10*	*10*	*10*	*10*	*10*	*10*
4 Acylated Ghrelin	Mean	**0**	**0.55**	**0.78**	**1.19**	**1.44**	**1.63**
	±SD	0	0.38	0.37	0.51	0.42	0.43
	n=	*10*	*10*	*10*	*10*	*10*	*10*
5 Control	Mean	**0**	**0.12**	**-0.09**	**0.21**	**0.29**	**0.38**
	±SD	0	0.28	0.31	0.28	0.37	0.31
	n=	*10*	*10*	*10*	*10*	*10*	*10*

Figure 3: Mean Group Cumulative Body Weight (BW) Gain Values (g) of 129Sv Mice Throughout the 6-day Treatment Period.

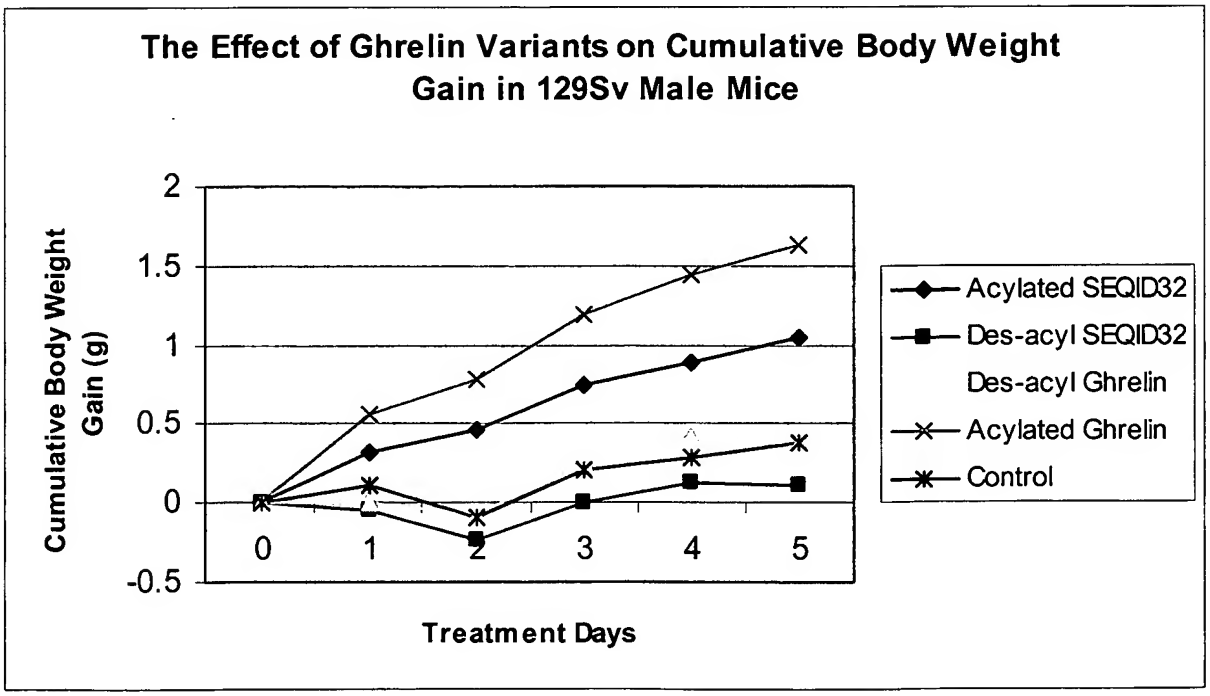


Table 6: Mean (± SD) Group Daily Food Consumption Values (g) of 129Sv Mice Throughout the 5-day Treatment Period.

Group		Study Day No											
		Pre-Test Conditioning Period					Treatment Period						
	Day	-4 to -3	-3 to -2	-2 to -1	-1 to 0	Daily FC Gain	0	0 to 1	1 to 2	2 to 3	3 to 4	4 to 5	Daily FC Gain
1 Acylated SEQID 32	Mean	**3.8**	**3.8**	**4.4**	**3.8**	**3.95**	**3.8**	**4.3**	**3.8**	**4.3**	**4.1**	**4.3**	**4.16**
	±SD	0.7	0.5	0.5	0.5		*0.5*	0.6	0.6	0.5	0.5	0.4	
	n=	*10*	*10*	*10*	*10*	4	*10*	*10*	*10*	*10*	*10*	*10*	5
2 Des-acyl SEQID 32	Mean	**3.9**	**4.0**	**3.9**	**3.7**	**3.87**	**3.7**	**3.3**	**3.4**	**3.6**	**3.7**	**3.6**	**3.52**
	±SD	0.3	0.5	0.6	0.6		*0.6*	0.9	0.7	0.4	0.3	0.3	
	n=	*10*	*10*	*10*	*10*	4	*10*	*10*	*10*	*10*	*10*	*10*	5
3 Des-acyl Ghrelin	Mean	**3.9**	**3.8**	**4.0**	**3.9**	**3.9**	**3.9**	**3.8**	**3.3**	**4.1**	**4.0**	**3.8**	**3.80**
	±SD	0.5	0.6	0.5	0.6		*0.6*	0.4	0.5	0.5	0.4	0.4	
	n=	*10*	*10*	*10*	*10*	4	*10*	*10*	*10*	*10*	*10*	*10*	5
4 Acylated Ghrelin	Mean	**3.8**	**3.9**	**4.3**	**3.9**	**3.97**	**3.9**	**4.4**	**4.0**	**4.5**	**4.5**	**4.4**	**4.36**
	±SD	0.4	0.5	0.4	0.6		*0.60*	0.5	0.5	0.5	0.4	0.9	
	n=	*10*	*10*	*10*	*10*	4	*10*	*10*	*10*	*10*	*10*	*10*	5
5 Control	Mean	**4.2**	**3.7**	**4.0**	**3.7**	**3.9**	**3.7**	**3.9**	**3.2**	**3.7**	**3.8**	**3.8**	**3.68**
	±SD	0.6	0.8	0.3	0.6		0.6	0.5	0.5	0.5	0.5	0.5	
	n=[(a)]	*10*	*10*	*10*	*10*	4	*10*	*10*	*10*	*10*	*10*	*10*	5

Figure 4: Mean Group Daily Food Consumption Values (g) of 129Sv Mice Throughout the Entire 10-days Observation Period, Including the 4-day pre-conditioning period and the 5-day Treatment Period.

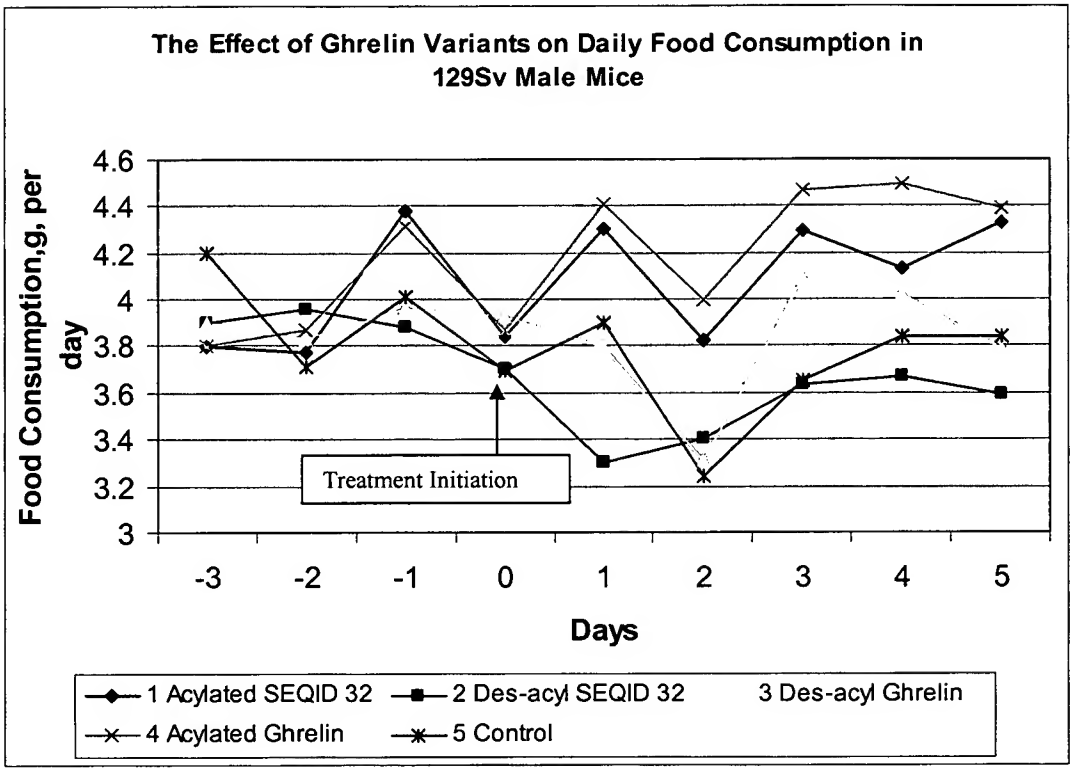
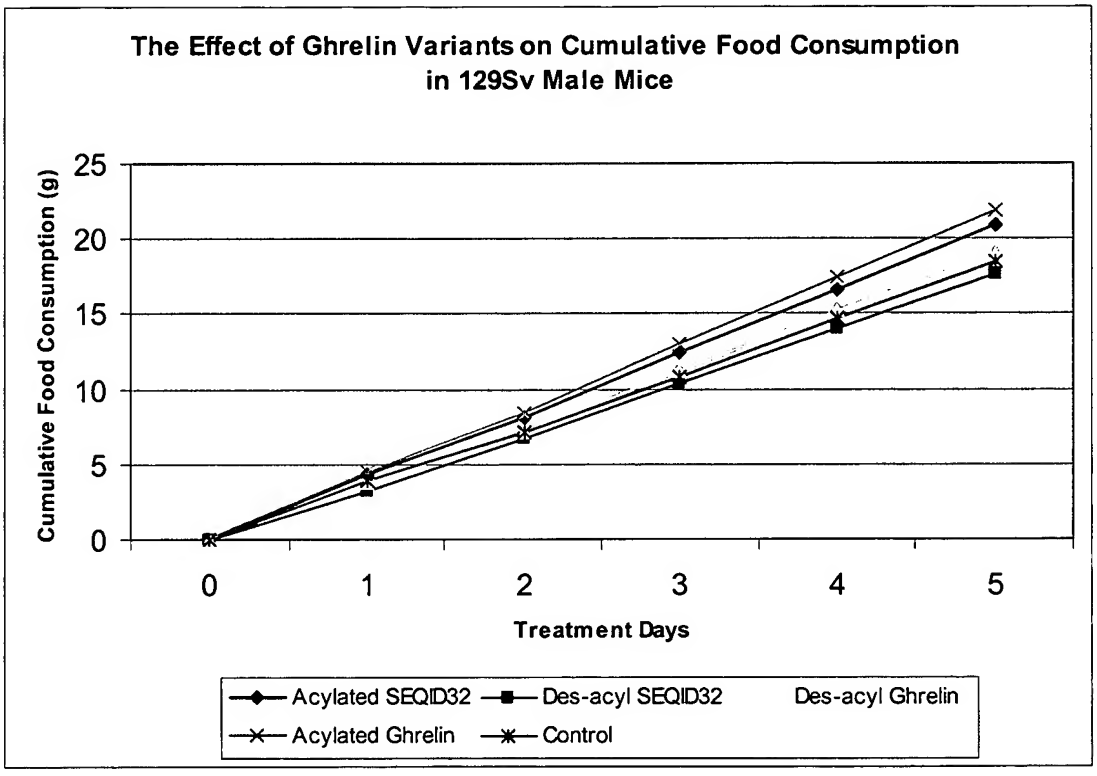


Table 7: Mean (± SD) Group Cumulative Food Consumption (FC) Values (g) of 129Sv Throughout the Entire 5-day Treatment Period.

Group		Study Day No Treatment Period					
	Day	0 to 1	0 to 2	0 to 3	0 to 4	0 to 5	0 to 6
1 Acylated SEQID 32	Mean	0	4.25	8.07	12.36	16.49	20.82
	±SD	0	0.59	1.10	1.52	1.9	2.28
	n=	10	10	10	10	10	10
2 Des-acyl SEQID 32	Mean	0	3.27	6.67	10.3	13.97	17.56
	±SD	0	0.87	1.17	1.25	1.43	1.64
	n=	10	10	10	10	10	10
3 Des-acyl Ghrelin	Mean	0	3.79	7.1	11.19	15.21	19
	±SD	0	0.43	0.80	1.20	1.47	1.76
	n=	10	10	10	10	10	10
4 Acylated Ghrelin	Mean	0	4.4	8.39	12.85	17.34	21.73
	±SD	0	0.52	0.98	1.40	1.72	2.32
	n=	10	10	10	10	10	10
5 Control	Mean	0	3.9	7.14	10.79	14.63	18.47
	±SD	0	0.54	0.96	1.46	1.81	2.18
	n=	10	10	10	10	10	10

Figure 5: Mean Group Cumulative Food Consumption Values (g) of 129Sv Mice Throughout the Entire 5-day Treatment Period.



visual-evoked brain activity results from the passive integration of auditory and visual inputs by multisensory neurons in the brain[3,21]. Such integrative effects are minimal when stimuli are separated by 100 ms or more. Because the present design used intervals of 100–300 ms between the auditory cue and visual target, the spatially specific facilitation of visual processing that we observed cannot be explained by passive multisensory integration alone. Instead, the improvement in d' for detection of the visual target can be ascribed to an involuntary orienting of attention to the location of the preceding auditory cue.

It has also been found that a spatially congruent, temporally overlapping sound improved response accuracy (per cent correct) for discriminating the orientation of a 'T' that was surrounded by distractor 'T's but not for discriminating the orientation of a line that was surrounded by distractor lines[22]. This latter, null effect indicated that visual processing at early, feature-extraction levels may not be altered by concomitant sounds. We found, however, that a preceding sound increased perceptual sensitivity (d') for simple luminance detection under conditions of brief, masked target presentations. This pattern of results is diagnostic of an attention effect upon the early perceptual processing of the spatially congruent visual target[15,18–20]. This cross-modal facilitation brought about by involuntary spatial attention may be a fundamental operation for enhancing the perceptual salience of natural stimuli in a multisensory world. □

Methods

Participants

Fifteen paid volunteers (8 women) between the ages of 19 and 30 years participated in experiment 1, and eighteen paid volunteers (9 women) between the ages of 19 and 37 participated in experiment 2. Informed, written consent was obtained from each participant.

Stimuli and apparatus

The experiment was conducted in a dimly lit, sound-attenuated chamber. The background sound level within the chamber was 32 dB(A). Participants sat in the chamber and faced a fixation point that was mounted at the centre of a 2.4-m horizontal arc. Loudspeakers mounted on the arc were positioned 38° to the left and right of the central fixation point (see Fig. 1a). A visual display was situated directly below each loudspeaker. Each light display contained four red light-emitting diodes (LEDs) arranged in a 1° square and one green LED positioned at the centre of the square. The distance between the centre of the loudspeaker cone and the centre of the light display was 2°, which is smaller than the minimum audible angle in the far periphery[23]. The auditory cue was a 76-dB (A) burst of broadband (500–5,000 Hz) 'pink' noise delivered from one of the two loudspeakers. The visual target was a flash of the green LED on either side of the fixation point. The visual mask was a simultaneous flash of all four red LEDs on either side of the fixation point. The luminance of the mask was 150 cd m^{-2}, and the luminance of the target varied between 20 and 100 cd m^{-2}. The luminance of the background was 2 cd m^{-2}.

Procedure

Each trial began with a 2,000-ms fixation period, during which time the fixation point, loudspeakers and light displays were all visible. Following the fixation period, an 83-ms cue period occurred during which a noise appeared with equal probability from either the left or right speaker. Then, after a random 100 to 300 ms (rectangular distribution), a 60-ms target period occurred. On half of the trials, the target appeared with equal probability on either the left or right side during the target period (target-present trials). On the other half of the trials, no target appeared during the target period (target-absent trials). Immediately following the target period, a mask was presented for 100 ms to either the left or right of fixation. In addition to masking the visual target, the mask indicated the location at which the target may have occurred. Participants were informed that the target and mask never appeared at different locations, and hence their present/absent judgement should be based solely on the masked location. The cue and mask appeared at the same location on 50% of the trials ('valid' trials) and at opposite locations on the other 50% of the trials ('invalid' trials). Thus, the noise cue provided no information about either the location of the mask or whether the target would be present or absent.

Each subject participated in a practice session followed by 15 experimental blocks. The intensity of the target was adjusted during the practice session so that each subject's hit rate was between 70% and 80%. Further adjustments were made between blocks if the subject's hit rate fell outside the 70–80% interval. Each block consisted of 28 valid trials and 28 invalid trials. In experiment 2, there were 14 additional trials per block on which no target or mask occurred. These catch trials were added to reduce anticipatory responses. Participants responded by pressing a button with their left or right thumb on alternating blocks.

Received 8 May; accepted 27 July 2000.

1. Bertelson, P. & Aschersleben, G. Automatic visual bias of perceived auditory location. *Psychon. Bull. Rev.* 5, 482–489 (1998).
2. Stein, B. E., London, N, Wilkinson, L. K. & Price, D. D. Enhancement of perceived visual intensity by auditory stimuli: A psychophysical analysis. *J. Cog. Neurosci.* 8, 497–506 (1996).
3. Stein, B. E. & Meredith, M. A. *The Merging of the Senses* (MIT Press, Cambridge, MA, 1993).
4. Welch, R. B. & Warren, D. H. in *Handbook of Perception and Human Performance* Vol. 1 (eds Boff, K. R., Kaufman, L. & Thomas, J. P.) 251, 25–36 (Wiley, New York, 1986).
5. McDonald, J. J. & Ward, L. M. Involuntary listening aids seeing: Evidence from human electrophysiology. *Psychol. Sci.* 11, 167–171 (2000).
6. Schmitt, M., Postma, A. & de Haan, E. Interactions between exogenous auditory and visual spatial attention. *Q. J. Exp. Psychol. A* 53, 105–130 (2000).
7. Spence, C. & Driver, J. Audiovisual links in exogenous covert spatial orienting. *Percept. Psychophys.* 59, 1–22 (1997).
8. Mondor, T. A. & Amirault, K. J. Effect of same- and different-modality spatial cues on auditory and visual target identification. *J. Exp. Psychol. Hum. Percept. Perform.* 24, 745–755 (1998).
9. Ward, L. M., McDonald, J. J. & Golestani, N. in *Visual Attention* (ed. Wright, R.) 232–268 (Oxford Univ., New York, 1998).
10. Ward, L. M. Supramodal and modality-specific mechanisms for stimulus-driven shifts of auditory and visual attention. *Can. J. Exp. Psychol.* 48, 242–259 (1994).
11. Ward, L. M., McDonald, J. J. & Lin, D. On asymmetries in crossmodal spatial attention orienting. *Percept. Psychophys.* 62, 1258–1264 (2000)
12. Driver, J. & Spence, C. Attention and the crossmodal construction of space. *Trends Cog. Sci.* 2, 254–262 (1998).
13. Driver, J. & Spence, C. Crossmodal attention. *Curr. Opin. Neurobiol.* 8, 245–253 (1998).
14. Sperling, G. & Dosher, B. A. in *Handbook of Perception and Performance* Vol. 1 (eds Boff, K. R., Kaufman, L. & Thomsas, J. P.) Ch. 2.1, 2–65 (Wiley, New York, 1986).
15. Shiu, L. & Pashler, H. Negligible effect of spatial precuing on identification of single digits. *J. Exp. Psychol. Hum. Percept. Perform.* 20, 1037–1054 (1994).
16. Pashler, H. E. *The Psychology of Attention* (MIT Press, Cambridge, MA, 1998).
17. Green, D. M. & Swets, J. A. *Signal Detection Theory and Psychophysics* (Wiley, New York, 1966).
18. Luck, S. J., Hillyard, S. A., Mouloua, M. & Hawkins, H. L. Mechanisms of visual-spatial attention: Resource allocation or uncertainty reduction? *J. Exp. Psychol. Hum. Percept. Perform.* 22, 725–737 (1996).
19. Luck, S. J. & Thomas, S. J. What variety of attention is automatically captured by peripheral cues? *Percept. Psychophys.* 61, 1424–1435 (1999).
20. Santee, J. L. & Egeth, H. E. Do reaction time and accuracy measure the same aspects of letter recognition? *J. Exp. Psychol. Hum. Percept. Perform.* 8, 489–501 (1982).
21. Meredith, M. A., Nemitz, J. W. & Stein, B. E. Determinants of multisensory integration in superior colliculus neurons. I. Temporal factors. *J. Neurosci.* 7, 3215–3229 (1987).
22. Dufour, A. Importance of attentional mechanisms in audiovisual links. *Exp. Brain. Res.* 126, 215–222 (1999).
23. Mills, A. W. On the minimum audible angle. *J. Acoust. Soc. Am.* 30, 237–246 (1958).

Acknowledgements

We thank D. Heraldez for research assistance, M. M. Marlow for computer assistance and L. Anllo-Vento for comments on the manuscript. This work was supported by grants from the National Institute of Health.

Correspondence and requests for materials should be addressed to J.J.M. (e-mail: jmcd@sfu.ca).

Ghrelin induces adiposity in rodents

Matthias Tschöp, David L. Smiley & Mark L. Heiman

Endocrine Research and BioResearch Technologies & Proteins, Lilly Research Laboratories, A Division of Eli Lilly and Company, Lilly Corporate Center, Drop Code 0545, Indianapolis, Indiana 46285, USA

The discovery of the peptide hormone ghrelin, an endogenous ligand for the growth hormone secretagogue (GHS) receptor[1,2], yielded the surprising result[3] that the principal site of ghrelin synthesis is the stomach and not the hypothalamus. Although ghrelin is likely to regulate pituitary growth hormone (GH) secretion[3,4] along with GH-releasing hormone and somatostatin, GHS receptors have also been identified on hypothalamic neurons[5] and in the brainstem[6]. Apart from potential paracrine effects, ghrelin may thus offer an endocrine link between stomach, hypothalamus and pituitary, suggesting an involvement in regulation of energy balance. Here we show that peripheral daily administration of ghrelin caused weight gain by reducing fat utilization in mice and rats. Intracerebroventricular administration of ghrelin

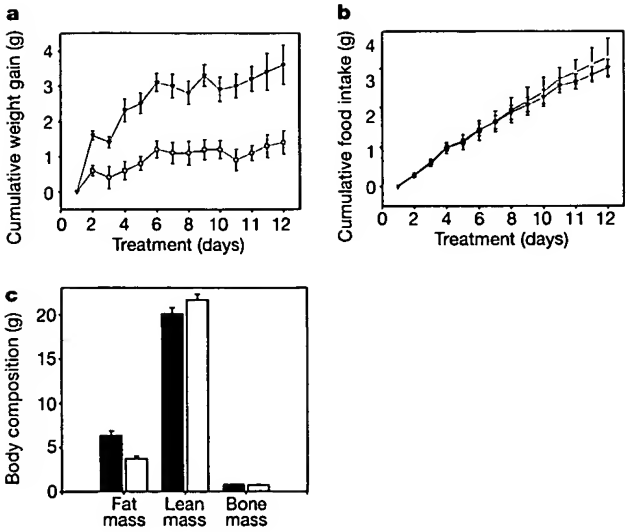


Figure 1 Ghrelin-stimulated adiposity in mice. **a**, Ghrelin induces body weight gain in male wild-type mice ($n = 10$ per group, $P = 0.0001$). Mice treated once daily for two weeks with ghrelin (2.4 μmol kg^{-1}, subcutaneously) gained 13.9% of their initial body weight (24.4 ± 1.0 g), while vehicle-injected control animals gained 5.6% of their initial body weight (25.1 ± 1.0 g). **b**, Ghrelin treatment did not change food intake rate in wild-type mice. **c**, Body composition of wild-type mice was measured by DXA after two weeks of treatment with ghrelin (2.4 μmol kg^{-1}, daily subcutaneously) or vehicle ($n = 10$ per group). Mice treated with ghrelin had a greater fat mass (6.34 ± 0.50 g) than vehicle-injected control animals (3.72 ± 0.29 g, $P = 0.002$). Symbols or bars represent the mean ± the standard error of the mean (s.e.m.). Filled symbols or bars, ghrelin treated; empty symbols or bars, controls.

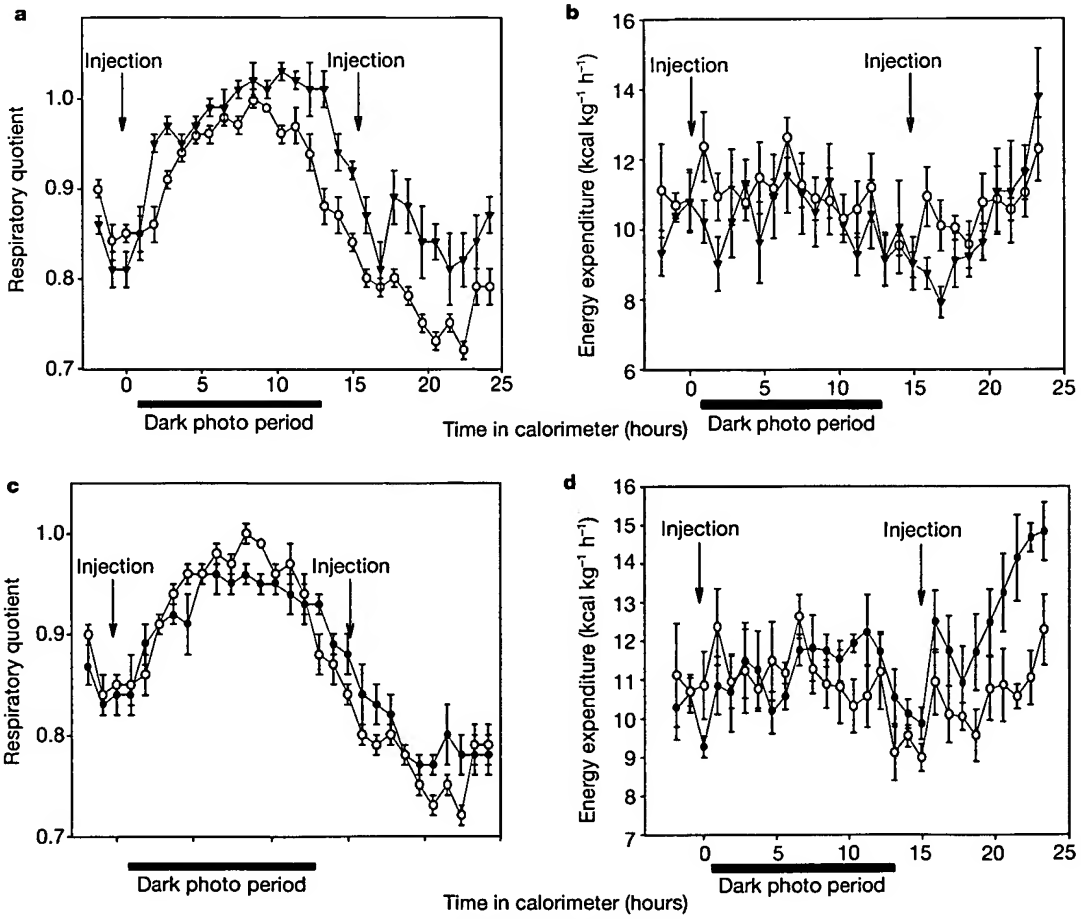


Figure 2 Differential effects of ghrelin and growth hormone on respiratory quotient (RQ) and energy expenditure (EE) in mice. **a**, Ghrelin increased RQ in wild-type mice after each peripheral administration ($P = 0.008$, each group $n = 5$). **b**, Ghrelin did not change total EE after peripheral application in mice. **c**, Growth hormone (8 mg kg^{-1}, subcutaneous) did not change RQ in wild-type mice (each group $n = 5$, $P = 0.67$) and **d**, did not significantly change total daily EE ($P = 0.18$), but increased EE during the light photoperiod ($P = 0.02$). Symbols represent the mean ± s.e.m. Filled triangles, ghrelin treated; filled circles, growth hormone treated; open circles, controls.

generated a dose-dependent increase in food intake and body weight. Rat serum ghrelin concentrations were increased by fasting and were reduced by re-feeding or oral glucose administration, but not by water ingestion. We propose that ghrelin, in addition to its role in regulating GH secretion, signals the hypothalamus when an increase in metabolic efficiency is necessary.

We synthesized rat ghrelin and assessed bioactivity by measuring GH release from primary pituitary cells (data not shown). Synthetic rat ghrelin (2.4 μmol kg^{-1} d^{-1}) or vehicle (phosphate buffered saline, PBS) was injected subcutaneously in male wild-type mice (129SV strain) once daily for two weeks. Body weight was significantly increased as compared to vehicle-injected mice (Fig. 1a), although ghrelin did not induce hyperphagia after once daily peripheral administration (Fig. 1b). Analysis of body composition by dual energy X-ray absorptiometry (DXA) after two weeks of ghrelin treatment revealed a significant gain in fat mass, but no change in lean body mass and bone mass (Fig. 1c), bone area (P = 0.41) or body length (P = 0.2) (data not shown), indicating absence of linear growth stimulation. As the fat gain seemed not to be the result of hyperphagia, we investigated whether ghrelin altered energy expenditure (EE) or decreased the proportion of fuel derived from fat.

Single subcutaneous ghrelin injections (2.4 μmol kg^{-1}) during the light photoperiod (resting phase for rodents) and the dark photoperiod (active feeding phase) induced an increase of the respiratory quotient (RQ) (P = 0.001, Fig. 2a). Such increased utilization of carbohydrate and reduced utilization of fat to meet energy requirements[7] is congruent with the observed increase in body fat. Neither EE (Fig. 2b) nor locomotor activity (vehicle-injected mice: 70.8 ± 9.6 beam-breaks h^{-1}; ghrelin-treated mice: 69.2 ± 1.9 beam-breaks h^{-1}; n = 5 per group, P = 0.9) were changed after peripheral application of ghrelin. A selective RQ increase without concomitant increases in carbohydrate intake is unusual and may reflect reduced sympathetic nervous system activity[8]. In addition, direct stimulation of hypothalamic areas can induce a selective change in RQ[9].

To ascertain the extent that these metabolic data are a consequence of GH release, we injected wild-type mice with GH (8 mg kg^{-1}, subcutaneously). GH did not change RQ during the dark or the light photoperiods (Fig. 2c). In contrast, a single injection of GH significantly increased EE during the light period, reflecting an enhanced energy utilization (Fig. 2d) with no significant alterations in locomotor activity (GH-treated mice: 75.1 ± 12.5 beam-breaks h^{-1}; vehicle-injected mice: 70.8 ± 9.6 beam-breaks h^{-1}; n = 5 per group, P = 0.9). Therefore, induction of a positive energy balance by ghrelin appears unlikely to be caused by its ability to stimulate GH secretion. Daily fat utilization of ghrelin-treated mice (30.6 ± 10.5 kcal kg^{-1} d^{-1}) was significantly less than that measured for GH-treated mice (68.8 ± 8.5 kcal kg^{-1} d^{-1}, P = 0.024) or vehicle-injected controls (65.6 ± 5.6 kcal kg^{-1} d^{-1}, P = 0.038). Ghrelin-treated mice gained 29.3 kcal kg^{-1} d^{-1} of fat (fat intake − fat utilization) while vehicle-injected (control) and GH-treated mice lost 5.7 kcal kg^{-1} d^{-1} and 7.2 kcal kg^{-1} d^{-1} of fat. Ghrelin-treated mice also tended to increase daily carbohydrate utilization (130.9 ± 13.6 kcal kg^{-1} d^{-1}, P > 0.17) as compared to both controls (103.5 ± 7.7 kcal kg^{-1} d^{-1}) and GH-treated mice (108.4 ± 8.17 kcal kg^{-1} d^{-1}).

These data expand the role of ghrelin from a GHS to a hormone that participates in regulating energy balance. Some GHSs are reported to stimulate food intake[10,11], and that orexigenic potential may be dependent on both dose and half-life. The described effects may not be mediated by GH, which is known to be lipolytic rather

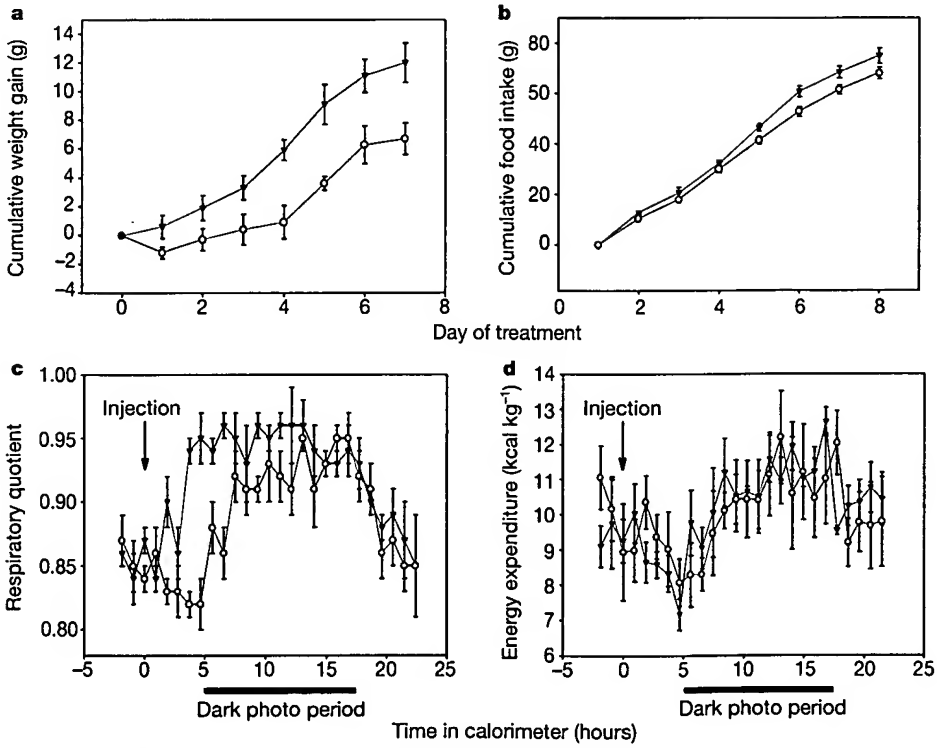


Figure 3 Ghrelin induced a positive energy balance in dwarf rats deficient in growth hormone. **a**, Ghrelin (4.5 μmol kg^{-1} daily, subcutaneous) increased body weight gain in female dwarf rats by 12.1% of their initial weight (98.9 ± 1.1g) when compared to vehicle-injected dwarf rats (+6.6%, initial weight: 101.3 ± 3.7 g, P = 0.01, each group n = 8). **b**, Ghrelin tended to increase food intake in dwarf rats (P = 0.1). **c**, Ghrelin (4.5 μmol kg^{-1}) increased RQ in GH-deficient dwarf rats after a single subcutaneous injection (n = 5 per group, P = 0.002). **d**, Ghrelin did not change total daily EE in GH-deficient rats, but decreased EE within the first three hours after administration. Symbols represented the mean ± s.e.m. Filled triangles, ghrelin treated; open circles, controls.

than lipogenic[12,13]. Further evidence that ghrelin-induced adiposity is independent from ghrelin-mediated GH release was obtained from our studies using GH-deficient dwarf rats[14]. An increase in body weight of female dwarfs was observed (Fig. 3a) during one week of ghrelin treatment (4.5 μmol kg^{-1} d^{-1}, subcutaneously). No significant increase in food intake was found, although there was a trend to overeat (Fig. 3b). Like wild-type mice treated with ghrelin, RQ was increased after a single peripheral ghrelin injection (Fig. 3c). Energy expenditure (Fig. 3d) or locomotor activity (data not shown) did not change.

The GHS/ghrelin receptor is present in neuropeptide Y (NPY) neurons[5,15] and central application of growth hormone-releasing peptide-6 (GHRP-6) induces increased expression of c-Fos protein (an index of cellular activation) in the same neurons[16,17]. NPY is one of the most effective molecules that induce body weight gain and is likely to mediate the metabolic physiology we observed. To investigate whether ghrelin requires NPY to stimulate fat accretion, we injected NPY-deficient mice[18] with ghrelin (2.4 μmol kg^{-1} d^{-1}) or vehicle for one week. Body weight gain similar to wild-type mice was observed ($P = 0.007$) after one week for ghrelin-treated NPY-deficient mice (+1.34 ± 0.30 g) as compared to controls (−0.14 ± 0.36 g). A small but significant ($P = 0.032$) increase in food intake was also found in ghrelin-treated NPY-deficient mice (cumulative food intake of ghrelin-treated NPY-deficient mice after one week: 23.7 ± 1.7 g, controls: 17.6 ± 2.0 g, $n = 10$ per group). We conclude that the presence of NPY is not obligatory for the ghrelin-stimulated fat accretion.

To determine whether ghrelin-induced adiposity is centrally mediated, we administered ghrelin at very low doses (~1,000-fold less than peripheral administration) intracerebroventricularly for one week in normal male adult rats ($n = 5$ per group) using mini-osmotic pumps. Continuous central ghrelin administration yielded a dose-dependent, highly significant increase in body weight (Fig. 4a), (1.2 nmol kg^{-1} d^{-1}: +26.7 ± 10.9 g week^{-1} ($P = 0.006$); 12 nmol kg^{-1} d^{-1}: +37.8 ± 4.8 g week^{-1} ($P < 0.001$); controls (water): +8.6 ± 5.0 g week^{-1}), enhanced food intake (Fig. 4b), (1.2 nmol kg^{-1} d^{-1}: 189.9 ± 18.5 g week^{-1} ($P = 0.002$); 12 nmol kg^{-1} d^{-1}: 204.36 g week^{-1} ($P < 0.001$); controls: 149.5 ± 4.4 g week^{-1}), and stimulated mean 24-hour respiratory quotient (Fig. 4c), 1.2 nmol kg^{-1} d^{-1}: 0.957 ± 0.018 ($P = 0.026$); 12 nmol kg^{-1} d^{-1}: 0.981 ± 0.007 ($P = 0.02$); controls: 0.907 ± 0.006). No differences in energy expenditure (Fig. 4d), ($P = 0.34$) or locomotor activity ($P > 0.5$) were recorded.

To examine whether endogenous ghrelin is influenced by feeding state, we measured circulating ghrelin levels in normal male Sprague–Dawley rats after 48 hours of fasting, fasting followed by re-feeding for 12 hours, and during *ad libitum* feeding. Serum ghrelin levels were increased in fasted animals (2.86 ± 28 ng ml^{-1}, $P < 0.001$) and reduced to levels of *ad libitum* fed rats (1.26 ± 14 ng ml^{-1}) after re-feeding (0.95 ± 5 ng ml^{-1}, $n = 9$ per group) (Fig. 5a). To investigate whether circulating ghrelin levels are regulated by stomach filling or nutrient intake, we compared oral administration of water (5 ml) with oral gavage of 50% dextrose in water (5 ml) (Fig. 5b and c). Stomach filling with water did not change ghrelin levels, but filling with dextrose significantly reduced serum ghrelin levels ($P = 0.001$).

Thus ghrelin, an endogenous ligand for the GHS-R, induces a positive energy balance in rodents by decreasing fat utilization

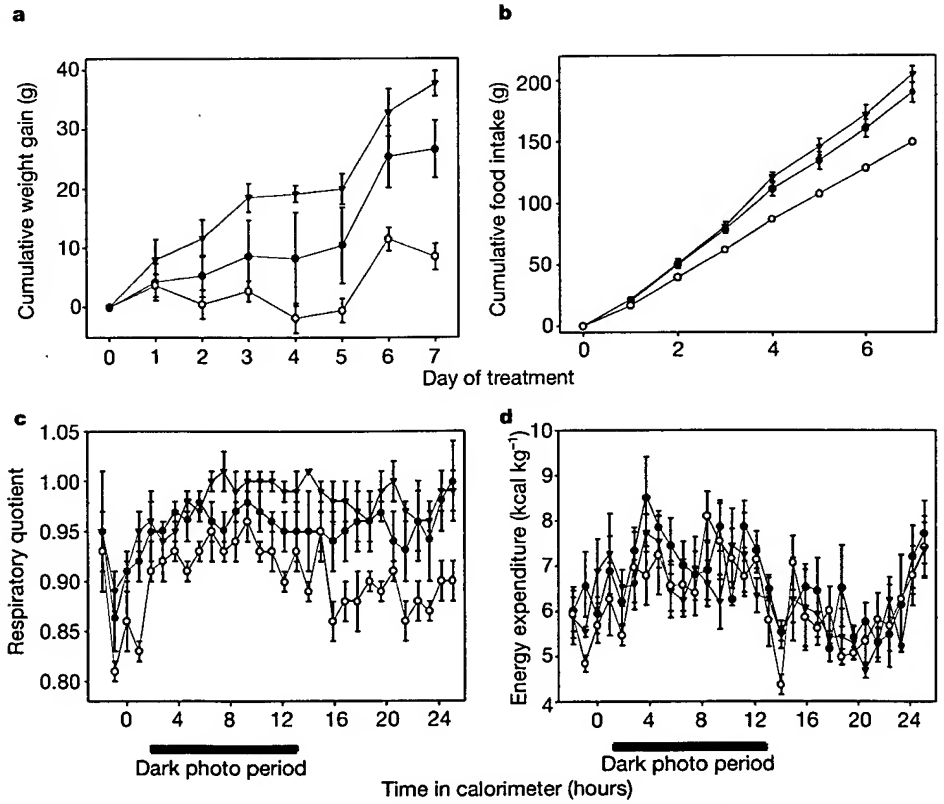


Figure 4 Central effects of ghrelin on energy balance in adult male Sprague–Dawley rats. **a**, Dose-dependent weight gain during continuous intracerebroventricular (ICV) administration of ghrelin (1.2 nmol kg^{-1} d^{-1} and 12 nmol kg^{-1} d^{-1}) for 7 days ($n = 5$ per group). **b**, Dose-dependent hyperphagia during ICV administration of ghrelin (1.2 nmol kg^{-1} d^{-1} and 12 nmol kg^{-1} d^{-1}) for 7 days ($n = 5$ per group). **c**, Dose-dependently increased RQ during continuous ICV administration of ghrelin (1.2 nmol kg^{-1} d^{-1} and 12 nmol kg^{-1} d^{-1}) for 7 days ($n = 5$ per group). **d**, Continuous ICV administration of ghrelin (1.2 nmol kg^{-1} d^{-1}and 12 nmol kg^{-1} d^{-1}) did not alter energy expenditure of adult rats ($n = 5$ per group). Symbols represented the mean ± s.e.m. Filled circles, 1.2 nmol kg^{-1} d^{-1} ghrelin ICV; filled triangles, 12 nmol kg^{-1} d^{-1} ghrelin ICV; open circles, controls.

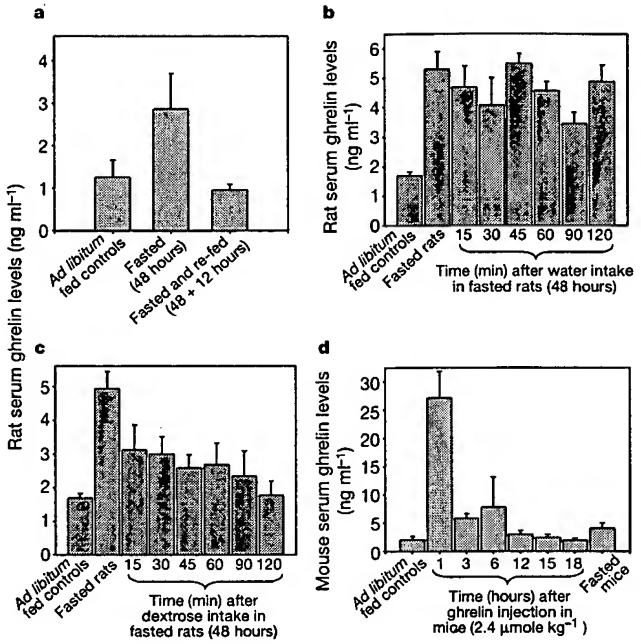


Figure 5 Circulating ghrelin levels respond to feeding state in adult male Sprague–Dawley rats. **a**, Influence of fasting and re-feeding on serum ghrelin levels. Each bar represents mean ± s.e.m. of 9 animals per group. **b**, Time course of serum ghrelin levels in rats (5 animals per group) after stomach filling with water (5 ml) in comparison to levels of fasted and *ad libitum* fed controls. **c**, Time course of serum ghrelin levels in rats (5 animals per group) after stomach filling with 50% dextrose solution (5 ml) in comparison to levels of fasted and *ad libitum* fed controls. **d**, Time course of serum ghrelin levels in mice (4 animals per group) after a single peripheral (subcutaneous) injection of 2.4 μmol ghrelin.

without significantly changing energy expenditure or locomotor activity. Serum ghrelin concentration in mice after peripheral (subcutaneous) administration of 2.4 $\mu mol\,kg^{-1}$ ghrelin was significantly greater than that measured during fasting only within the first hour after injection (Fig. 5d). This indicates that ghrelin has a short half-life. Rapid degradation might offer an explanation for lack of measurable hyperphagia following peripheral administration.

The dramatic diurnal RQ rhythm (Figs 2a and c, 3c and 4c) could be the consequence of a diurnal ghrelin rhythm responding to nutrient absorption postprandially and to an emptying stomach during the rodent's sleep period. Although the peptide is rapidly cleared after peripheral administration (Fig. 5d), it impressively induces hyperphagia after continuous central infusion of doses as low as $1.2\,nmol\,kg^{-1}\,day^{-1}$ (Fig. 4b), indicating a central mode of action. Ghrelin-induced metabolic changes led to an efficient metabolic state resulting in increased body weight and fat mass. These effects were GH-independent and did not require the presence of NPY. Similar data were observed after administration of growth hormone releasing peptide-2 (GHRP-2), a synthetic agonist of the GHS-R (data will be published elsewhere), indicating a GHS/ghrelin receptor-mediated mechanism. Further, a positive energy balance is necessary to maximize the anabolic actions of GH. As ghrelin stimulates both energy gain and GH secretion, it provides an integrated means to produce an anabolic state. Fasting increases ghrelin serum levels whereas re-feeding blunts this increase, suggesting a role for ghrelin in shifting to a more energy-efficient metabolism during starvation. Sugar intake, but not stomach expansion, decreases circulating ghrelin levels. Although ghrelin is undoubtedly a regulator of GH secretion, our data indicate that this new gastric hormone also signals hypothalamic regulatory centres controlling energy balance. Because ghrelin was detected in the hypothalamus by polymerase chain reaction after reverse transcription of RNA and by immunohistochemistry[3], we cannot rule out the possibility that stomach-released ghrelin regulates GH release whereas central ghrelin influences energy balance. Despite the mechanism, our data indicate that ghrelin may be a new link between the GH/IGF-1 (growth hormone/insulin-like growth factor-1) axis and the neuroendocrine regulation of energy balance. □

Methods

Animals

Wild-type mice (129SV strain) were purchased from Taconic Farms and NPY-knockout mice[18], which were originally provided to us by R. Palmiter, were raised for us at Taconic Farms. Eight-week old dwarf rats[14] were purchased at Harlan, UK. Animals were housed individually in a temperature-controlled environment (25 °C) with a 12-hour light and 12-hour dark (18.00–06.00) photoperiod. All mice had *ad libitum* access to pelleted mouse food and tap water. Calories provided by mouse diet (5008 PMI Nutrition International) were derived from 26.8% protein, 16.7% fat and 56.4% carbohydrate. Calories provided by rat diet (5001 PMI Nutrition International) were derived from 28.0% protein, 12.1% fat and 59.8% carbohydrate. Mice were between 10 and 13 weeks of age and were injected subcutaneously daily between 17.00 and 18.00 with 0.1 ml of PBS containing 0 or $2.4\,\mu mol\,kg^{-1}\,d^{-1}$ ghrelin over 13 days. Food intake and body weights were measured daily at 08.00. Mini-osmotic pumps (Alzet, Alza Pharmaceuticals) were implanted in three groups of five adult male Sprague-Dawley rats for intracerebroventricular (ICV) administration of ghrelin. Pumps were continuously delivering water, $1.2\,nmol\,kg^{-1}\,d^{-1}$ ghrelin or $12\,nmol\,kg^{-1}\,d^{-1}$ ghrelin for 7 days at $1.0\,\mu l\,h^{-1}$. The study protocols used in these experiments were approved by the Animal Care Committee of Eli Lilly and Co. All animal experiments were conducted in accordance with the principles and procedures outlined in the National Institute of Health (NIH) Guide for the Care and Use of Laboratory Animals.

Indirect calorimetry

Twenty-four-hour energy expenditure (EE) and respiratory quotient (RQ) were measured by indirect calorimetry as described[19], using an open circuit calorimetry system (Oxymax, Columbus Instruments Int. Corp.). RQ is the ratio of the volume of CO_2 produced (VCO_2) to the volume of O_2 consumed (VO_2). EE was calculated as the product of calorific value of oxygen (CV) and VO_2 per kilogram of body weight; where CV = 3.815 + 1.232(RQ) (ref. 20). Total calories expended were calculated to determine daily fuel utilization. To calculate the proportion of protein, fat and carbohydrate that is used during that 24-hour period, we used Flatt's proposal[21] and formulae as well as other derived constants[20]. Locomotor activity was measured by counting the number of times an animal broke a new light beam during each period of 24 hours in the calorimeter.

Body composition

Body composition was measured *in vivo* on day 14 of the treatment period by dual-energy X-ray absorptiometry (DXA) using a p-DEXA instrument (Norland, USA) as described previously[22].

Ghrelin

We synthesized rat ghrelin on an Advanced ChemTech 396 synthesizer and with 9-fluorenylmethoxycarbonyl (FMOC) amino acids and 50-minute diisopropylcarbodiimide (DIC)/1-hydroxibenzotriazole (HOBT) activated double couplings. FMOC-SER(Trt) was used in the couplings for Ser3. Following trityl deprotection using 1% TFA / 5% tri-isopropylsilane in methylene chloride (DCM), the Ser3-hydroxyl was acylated using excess octanoic acid and 1,3[(dimethylamino)propyl]-3-ethylcarbodiimide hydrochloride salt (EDAC) in the presence of 4-dimethylaminopyridine (DMAP). After removal of the N-terminal FMOC, a 2-hour cleavage was run using Reagent K. The precipitated peptide was washed with ethyl ether and dried in vacuum. The material was dissolved in aqueous acetic acid and purified over a 2.2 × 25 cm VydacC18 column using a gradient of 15% A to 55% B over 450 min (A = 0.1% TFA, B = 0.1% TFA / 50% CH_3CN). Five-minute fractions were collected while monitoring the ultraviolet at 214 nm (2.0A). The appropriate fractions were combined, frozen and lyophilized. We isolated mono-octanoylated ghrelin and tested for ability to release GH in a primary rat pituitary cell assay. Matrix-assisted laser desorption/ionization time-of-flight mass spectroscopy indicated a mass of 3313.85 g for the purified ghrelin, which was consistent with the theoretical molecular weight.

Bovine growth hormone

Bovine GH was prepared by recombinant DNA technology as described elsewhere[23].

Primary pituitary cell assay

Primary pituitary cells were prepared as described[24] and challenged with ghrelin for 15 min at 37 °C. Media was removed and rat GH was measured by radioimmunoassay.

Radioimmunoassays

Rat serum ghrelin levels were measured by a commercially available RIA (Phoenixpeptide) and rat GH level in media from primary pituitary cells was measured by a RIA kit (Amersham).

Statistics

Statistical analysis was performed using one way analysis of variance (ANOVA) with Tukey post-hoc testing using Sigmastat Software.

Received 7 July; accepted 11 September 2000.

1. McKee, K. K. *et al.* Molecular analysis of rat pituitary and hypothalamic growth hormone secretagogue receptors. *Mol. Endocrinol.* **11**, 415–423 (1997).
2. Howard, A. D. *et al.* A receptor in pituitary and hypothalamus that functions in growth hormone release. *Science* **273**, 974–977 (1996).
3. Kojima, M. *et al.* Ghrelin is a growth-hormone-releasing acylated peptide from stomach. *Nature* **402**, 656–660 (1999).
4. Hosoda, H., Kojima, M., Matsuo, H. & Kangawa, K. Purification and characterization of rat des-Gln14-ghrelin, a second endogenous ligand for the growth hormone secretagogue receptor. *J. Biol. Chem.* **275**, 21995–22000 (2000).
5. Guan, X. M. *et al.* Distribution of mRNA encoding the growth hormone secretagogue receptor in brain and peripheral tissues. *Brain Res.* **48**, 23–29 (1997).
6. Bailey, A. R., Von Englehardt, N., Leng, G., Smith, R. G. & Dickson, S. L. Growth hormone secretagogue activation of the arcuate nucleus and brainstem occurs via a non-noradrenergic pathway. *J. Neuroendocrinol.* **12**, 191–197 (2000).
7. Scheurink, A. J., Leuvenink, H., Benthem, B. & Steffens, A. B. Dexfenfluramine treatment influences plasma catecholamines and energy substrate metabolism in rats. *Physiol. Behav.* **53**, 879–887 (1993).
8. Snitker, S., Tataranni, P. A. & Ravussin, E. Respiratory quotient is inversely associated with muscle sympathetic nerve activity. *J. Clin. Endocrinol. Metab.* **83**, 3977–3979 (1998).
9. Atrens, D. M., Sinden, J. D., Penicaud, L., Devos, M. & Le Magnen, J. Hypothalamic modulation of energy expenditure. *Physiol. Behav.* **35**, 15–20 (1985).
10. Kuriyama, H., Hotta, M., Wakabayashi, I. & Shibasaki, T. A 6-day intracerebroventricular infusion of the growth hormone-releasing peptide KP-102 stimulates food intake in both non-stressed and intermittently-stressed rats. *Neurosci. Lett.* **282**, 109–112 (2000).
11. Okada, K. *et al.* Intracerebroventricular administration of the growth hormone-releasing peptide KP-102 increases food intake in free-feeding rats. *Endocrinology* **137**, 5155–5158 (1996).
12. Salomon, F., Cuneo, R. C., Hesp, R. & Sonksen, P. H. The effects of treatment with recombinant human growth hormone on body composition and metabolism in adults with growth hormone deficiency. *N. Engl. J. Med.* **321**, 1797–1803 (1989).
13. Vernon, R. G. GH inhibition of lipogenesis and stimulation of lipolysis in sheep adipose tissue: involvement of protein serine phosphorylation and dephosphorylation and phospholipase C. *J. Endocrinol.* **150**, 129–140 (1996).
14. Charlton, H. M. *et al.* Growth hormone-deficient dwarfism in the rat: a new mutation. *J. Endocrinol.* **119**, 51–58 (1988).
15. Willesen, M. G., Kristensen, P. & Romer, J. Co-localization of growth hormone secretagogue receptor and NPY mRNA in the arcuate nucleus of the rat. *Neuroendocrinology* **70**, 306–316 (1999).
16. Bailey, A. R. *et al.* Chronic central infusion of growth hormone secretagogues: effects on fos expression and peptide gene expression in the rat arcuate nucleus. *Neuroendocrinology* **70**, 83–92 (1999).
17. Luckman, S. M., Rosenzweig, I. & Dickson, S. L. Activation of arcuate nucleus neurons by systemic administration of leptin and growth hormone-releasing peptide-6 in normal and fasted rats. *Neuroendocrinology* **70**, 93–100 (1999).
18. Erickson, J. C., Clegg, K. E. & Palmiter, R. D. Sensitivity to leptin and susceptibility to seizures of mice lacking neuropeptide Y. *Nature* **381**, 415–421 (1996).
19. Chen, Y. & Heiman, M. L. Chronic leptin administration promotes lipid utilization until fat mass is greatly reduced and preserves lean mass of normal female rats. *Regul. Pept.* (in the press).
20. Elia, M. & Livesey, G. Energy expenditure and fuel selection in biological systems: the theory and practice of calculations based on indirect calorimetry and tracer methods. *World Rev. Nutr. Diet* **70**, 68–131 (1992).
21. Flatt, J. P. Assessment of daily and cumulative carbohydrate and fat balances in mice. *J. Nutr. Biochem.* **2**, 193–202 (1991).
22. Rose, B. S., Flatt, W. P., Martin, R. J. & Lewis, R. D. Whole body composition of rats determined by dual energy X-ray absorptiometry is correlated with chemical analysis. *J. Nutr.* **128**, 246–250 (1998).
23. Hsiung, H. M. & MacKellar, W. C. Expression of bovine growth hormone derivatives in *Escherichia coli* and the use of the derivatives to produce natural sequence growth hormone by cathepsin C cleavage. *Methods Enzymol.* **153**, 390–401 (1987).
24. Heiman, M. L., Nekola, M. V., Murphy, W. A., Lance, V. A. & Coy, D. H. An extremely sensitive in vitro model for elucidating structure-activity relationships of growth hormone-releasing factor analogs. *Endocrinology* **116**, 410–415 (1985).

Acknowledgements

We thank J. Caro, G. Cutler Jr, E. Ravussin, R. Al-Awar, C.J. Strasburger and A. Tashjian Jr for critical review, R. Palmiter for providing NPY-deficient mice and L. Craft, J. Baker, J. Bridwell, J. Jacobs, W.T. Johnson, P. Surface, F. Tinsley and T. Butler for technical assistance.

Correspondence and requests for materials should be addressed to M.T. (e-mail: Matthias_Tschoep@Lilly.com).

Repressor activity of Headless/Tcf3 is essential for vertebrate head formation

Cheol-Hee Kim*, Takaya Oda†, Motoyuki Itoh*, Di Jiang*, Kristin Bruk Artinger‡, Settara C. Chandrasekharappa†, Wolfgang Driever§ & Ajay B. Chitnis*

** Laboratory of Molecular Genetics, NICHD and † Genetics and Molecular Biology Branch, NHGRI, NIH, Bethesda, Maryland 20892, USA*
‡ Department of Cell Biology, Harvard Medical School, Boston, Massachusetts 02115, USA
§ University of Freiburg, Institute for Zoology, Hauptstrasse 1, D-79104 Freiburg, Germany

The vertebrate organizer can induce a complete body axis when transplanted to the ventral side of a host embryo[1] by virtue of its distinct head and trunk inducing properties. Wingless/Wnt antagonists secreted by the organizer have been identified as head inducers[2–4]. Their ectopic expression can promote head formation, whereas ectopic activation of Wnt signalling during early gastrulation blocks head formation[5–7]. These observations suggest that the ability of head inducers to inhibit Wnt signalling during formation of anterior structures is what distinguishes them from trunk inducers that permit the operation of posteriorizing Wnt signals[8]. Here we describe the zebrafish *headless* (*hdl*) mutant and show that its severe head defects are due to a mutation in T-cell factor-3 (Tcf3), a member of the Tcf/Lef family[9,10]. Loss of Tcf3 function in the *hdl* mutant reveals that *hdl* represses Wnt target genes. We provide genetic evidence that a component of the Wnt signalling pathway is essential in vertebrate head formation and patterning.

The *hdl* mutant was isolated as part of a screen for ethyl nitrosourea (ENU)-induced mutations that disrupt early neurogenesis in zebrafish[11]. Mutant embryos obtained from *hdl* heterozygous parents, however, display a weak phenotype and are characterized by a slight reduction in eye size. Their weak phenotype allowed a subset of homozygous *hdl* fish to be grown to adulthood. Here, *hdl* mutants refers to maternally and zygotically homozygous mutant

Ghrelin stimulation of growth hormone release and appetite is mediated through the growth hormone secretagogue receptor

Yuxiang Sun*†, Pei Wang*, Hui Zheng*, and Roy G. Smith*†‡§

*Huffington Center on Aging, Departments of †Molecular and Cellular Biology and ‡Medicine, Baylor College of Medicine, Houston, TX 77030

Edited by Richard D. Palmiter, University of Washington School of Medicine, Seattle, WA, and approved February 5, 2004 (received for review September 15, 2003)

Synthetic agonists of the growth hormone secretagogue receptor (GHSR) rejuvenate the pulsatile pattern of GH-release in the elderly, and increase lean but not fat mass in obese subjects. Screening of tissue extracts in a cell line engineered to overexpress the GHSR led to the identification of a natural agonist called ghrelin. Paradoxically, this hormone was linked to obesity. However, it had not been directly shown that the GHSR is a physiologically relevant ghrelin receptor. Furthermore, ghrelin's structure is significantly different from the synthetic agonist (MK-0677) used to expression-clone the GHSR. To address whether the GHSR mediates ghrelin's stimulatory effects on GH release and appetite, we generated *Ghsr*-null mice. In contrast to wild-type mice, acute treatment of *Ghsr*-null mice with ghrelin stimulated neither GH release nor food intake, showing that the GHSR is a biologically relevant ghrelin receptor. Nevertheless, *Ghsr*-null mice are not dwarfs; their appetite and body composition are comparable to that of wild-type littermates. Furthermore, in contrast to suggestions that ghrelin regulates leptin and insulin secretion, fasting-induced changes in serum levels of leptin and insulin are identical in wild-type and null mice. Serum insulin-like growth factor 1 levels and body weights of mature *Ghsr*-null mice are modestly reduced compared to wild-type littermates, which is consistent with ghrelin's property as an amplifier of GH pulsatility and its speculated role in establishing an insulin-like growth factor 1 set-point for maintaining anabolic metabolism. Our results suggest that chronic treatment with ghrelin antagonists will have little effect on growth or appetite.

In 1988, a reverse pharmacology approach was initiated to identify small molecules that would restore the amplitude of growth hormone (GH) pulsatility in the elderly (1). We elucidated the mechanism of action of a class of small, synthetic, GH-releasing peptides, and used this knowledge to develop nonpeptide mimetics (2–5). The mimetic MK-0677, when administered chronically to elderly subjects, resulted in sustained rejuvenation of the physiological profile of the growth hormone axis, and increased lean but not fat mass in obese subjects (6, 7). MK-0677 was also exploited to expression-clone the receptor involved (8); this orphan G protein-coupled receptor was named the GH secretagogue receptor (GHSR) (8). Besides the pituitary gland and hypothalamic areas that regulate GH release, the GHSR is expressed in brain centers that control appetite, pleasure, mood, biological rhythms, memory, and cognition (6, 9, 10).

Ghrelin and adenosine were identified as naturally occurring agonists for the orphan GHSR by fractionating and assaying animal tissue extracts in cell lines engineered to express the GHSR (11–13). Administration of ghrelin and adenosine to rats stimulates feeding, but only ghrelin stimulates GH release (12, 14). Accordingly, ghrelin more closely mimics MK-0677, and it was assumed that the GHSR is the ghrelin receptor. However, evidence has been presented to suggest the existence of receptor subtypes (15). Furthermore, as a 28-aa peptide containing a unique octanoyl modification (11), ghrelin is structurally different from MK-0677. Although molecular modeling studies that compared structural features assigned from proton NMR of MK-0677 and other synthetic GHSR ligands illustrated certain similarities with ghrelin, these studies did not precisely predict the receptor–ligand binding characteristics (16). To directly investigate a potentially significant physiological relationship between ghrelin and GHSR, we generated *Ghsr*-null (−/−) mice.

Materials and Methods

Generation of *Ghsr* Null Mice. In the targeting vector, a pGKneo cassette was used to replace a region from the *Pst*I site at 5′ of the coding exon-1 to the *Hin*dIII site in the coding exon-2 (Fig. 1). The targeting vector was linearized by *Bam*HI digestion and transfected into 129Sv embryonic stem (ES) cells by electroporation. The 1.1-kb *Not*I/*Bam*HI fragment at the 5′ end of the genomic clone was used as the 5′ external probe to select positive ES clones in Southern analysis. The appropriately targeted ES cells were injected into blastocysts derived from C57BL/6J. Southern analysis was later used in genotyping the offspring of heterozygous parents. Ten micrograms of mouse DNA was digested with either *Eco*RI or *Hin*dIII, electrophoresed on 0.8% agarose gel, transferred to the membrane, and hybridized with either the 5′ external probe or the exon probe. When the 5′ external probe was hybridized with *Eco*RI-digested DNA, a 14-kb fragment was produced from the wild-type allele. Because of the presence of an *Eco*RI site in the PGKneo cassette, a 6.7-kb fragment was produced from the mutant allele. To ensure the deletion of the coding region, an exon probe (a 1.1-kb *Pst*I/*Hin*dIII fragment encoding part of the first coding exon) was used. When *Hin*dIII-digested DNA was hybridized with the exon probe, the 2.3-kb *Hin*dIII fragment corresponding to the *Ghsr* exon was detected in *Ghsr* wild type (+/+) and heterozygote (+/−), but not in homozygote (−/−) mice. To confirm the precise integration of mutant fragment at both insertion sites, the long-template PCR was performed by using Expand Long Template PCR System (Boehringer Mannheim). For the 5′ long PCR fragment (4.5-kb): forward primer, 5′-GGGATGGGCACATGAATCTTTCTGGAAAGGGGG; reverse primer, 5′-GGAAAAGCGCCTCCCCTACCCGGTAGAATTC. For the 3′ long PCR fragment (6.0-kb): forward primer, 5′-CTTCTATCGCCTTCTTGACGAGTTCTTCTGAGG; reverse primer, 5′-GACCATCAGAGAGGATACACAGATTGGAAGC.

RT-PCR Analysis. Total RNA was isolated from individual mice. Twenty nanograms of total RNA was used in semiquantitative RT-PCR. The intron flanking primers are: forward, 5′-TATGGGTGTCGAGCGTCTT (in coding exon 1); reverse, 5′-GAGAATGGGGTTGATGGC (in coding exon 2).

This paper was submitted directly (Track II) to the PNAS office.

Abbreviations: GH, growth hormone; GHSR, GH secretagogue receptor; ES, embryonic stem; GHRH, GH-releasing hormone; IGF-1, insulin-like growth factor 1.

§To whom correspondence should be addressed. E-mail: rsmith@bcm.tmc.edu.

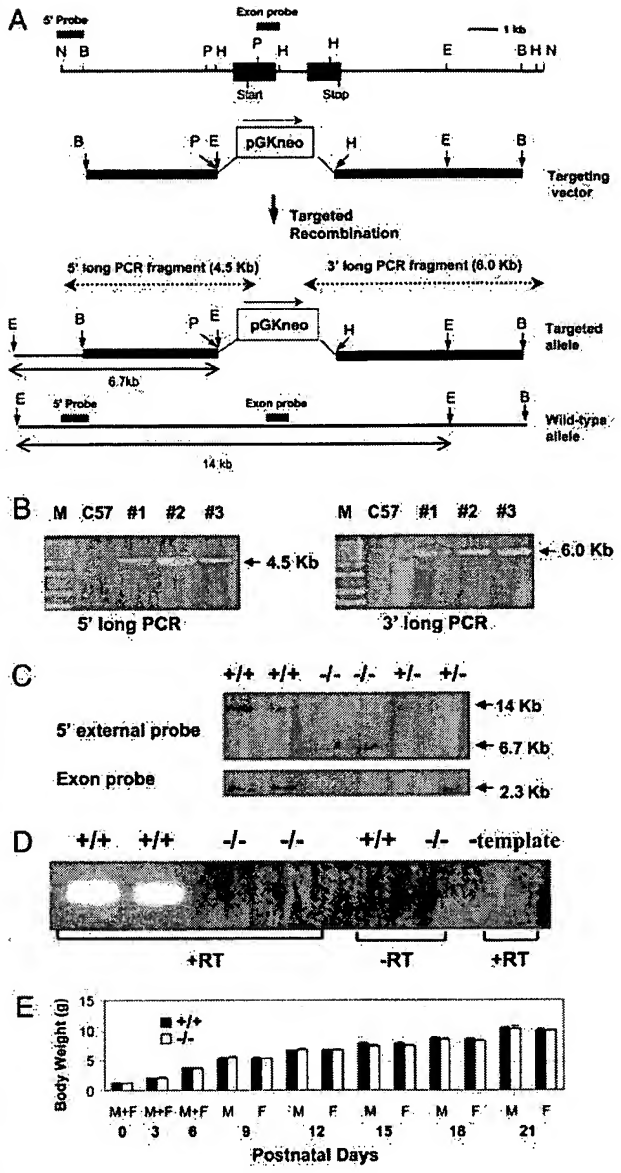


Fig. 1. (*A*) Restriction enzyme map of a mouse *Ghsr* genomic DNA clone, and the strategy for deriving *Ghsr*−/− mice by homologous recombination. The filled boxes represent the two coding exons. Restriction enzyme sites: N, *Not*I; B, *Bam*HI; P, *Pst*I; H, *Hin*dIII; E, *Eco*RI. The dotted lines show the long-template PCR products. (*B*) Long-template PCR analysis of F_1 founder mice. M, Marker; C57, C57BL/6J mice for control; 1, 2, and 3 are the three founder mice. (*C*) Southern blot analysis of the offspring from heterozygous mating. (*Upper*) DNA was digested with *Eco*RI then hybridized with 5′ probe. (*Lower*) DNA was digested with *Hin*dIII then hybridized with exon probe. The 5′ probe and exon probe are shown in *A*. +/+, wild-type; −/−, homozygote; +/−, heterozygote. (*D*) GHSR mRNA expression in pituitary as determined by RT-PCR. +RT, with reverse transcriptase; −RT, without reverse transcriptase; −template, with reverse transcriptase but without RNA template. (*E*) Body weights of pups at birth and postnatal days. Before sex can be distinguished (from day 0 to day 6), $n = 48$ for −/− and 51 for +/+. From day 9 to weaning, body weight data from male and female pups were collected separately.

Hormone Assays. All hormone assays were done in mature male mice. In fasting experiments, the fasting time was 48 h from 8 a.m. to 8 a.m. To measure GH, mice were injected i.p. with pentobarbital (50 mg/kg body weight); 15 min later, 100 μl of physiologic saline, either with or without 10 μg of ghrelin (Phoenix Pharmaceuticals, St. Joseph, MO), was injected i.p. Blood was collected by retro-orbital bleeding at 0, 5, and 15 min after saline/ghrelin. GH was measured in plasma samples by using rat GH EIA kit (American Laboratory Products, Windham, NH). Ten micrograms of MK-0677 (Merck Research Laboratories) and 10 μg of human GH-releasing hormone (GHRH, Phoenix Pharmaceuticals) were also tested in similar experimental setup. For other assays, blood was collected by either retro-orbital bleeding or tail vein bleeding. Serum was collected for measurements of: ghrelin (rat ghrelin RIA kit, Phoenix Pharmaceuticals); leptin (mouse leptin RIA kit, Linco Research Immunoassays, St. Joseph, MO); insulin (sensitive rat insulin RIA kit, Linco Research Immunoasays); insulin-like growth factor 1 (IGF-1) (rat IGF-1 RIA kit, Diagnostic Systems Laboratories, Webster, TX).

Effects of Acute Administration of Ghrelin on Appetite. Mice were injected i.p. with 100 μl of physiologic saline first and food intake was measured at 0.5 h after the saline injection (0–0.5 h). Later, the same mice were injected with 100 μl of physiologic saline containing 10 μg of ghrelin. Food intake was measured at 0.5 h and 1.0 h after the ghrelin injection to get the food intake of the first 0.5 h (0–0.5 h) and the second 0.5 h (0.5–1.0 h). One hour after the first ghrelin injection, ghrelin was reinjected, and the food intake during the next 0.5 h (0–0.5 h) was measured.

Body Composition. Bone density (bone mineral density and bone mineral content) and body composition (fat %) were measured by using the noninvasive technique of dual energy-x-ray absorptiometry (Lunar PIXI Mouse densitometer, Lunarcorp, Madison, WI). Fat and lean body mass were also measured by using a Minispec mq benchtop NMR spectrometer (Bruker Instruments) at the Yale Mouse Metabolic Phenotyping Center. The fat represents total fat, independent of where it is localized. The intensities of the fat, muscle, and free fluid were calculated automatically from the time domain [^{1}H]NMR signals by the instrument software and expressed in units of grams.

Body Weight and Food Intake Under Ad Libitum Condition. The experimental mice were individually caged and provided with ad libitum access to water and regular chow. Body weight and food intake were measured every other week at the same time of the day.

The Evaluation of Appetite During Fasting and Refeeding. The mice (12 weeks old) were weighed and chow was removed. Twenty-four hours later, the animals were weighed, then provided with a weighed amount of chow, and food intake was measured at 1, 2, 4, 6, 24, and 48 h; body weights were measured at 24 and 48 h.

Animals and Data Analysis. All experiments were conducted on N3 mice by backcrossing F_1 mice onto C57BL/6J mice for two generations. In all experiments, *Ghsr*-null mice (*Ghsr*−/−) were compared to wild-type littermates (*Ghsr*+/+). Mice were kept in a standard 7 a.m. to 7 p.m. light cycle (light off at 7 p.m.) facility, and fed with regular mouse chow. Mice were housed one per cage during the experiments. Data are presented as mean ± SEM in all figures. The number of subjects is indicated by n. Significant differences between the groups were evaluated by different ANOVA tests using SIGMASTAT 3.0 software. Two-way ANOVA test was used for Figs. 1*E*, 2, 3, 4, and 5. Figs. 1*E* and 4 *A* and *B* were also evaluated by two-way repeated-measures ANOVA. $P < 0.05$ was considered as statistical significance.

Results and Discussion

To generate *Ghsr*-null mice, a 15.3-kb *Ghsr* mouse genomic phage clone was used to characterize the *Ghsr* locus. A pGKneo cassette was inserted into the *Ghsr* locus to replace the entire coding exon 1 and part of the coding exon 2. The targeting vector consisted of 3.6- and 5.7-kb homologous regions of genomic

DNA at 5′ and 3′ of the selection cassette, respectively (Fig. 1*A*). After initial PCR analysis of all agouti mice, three positive F_1 founder mice were identified. To ensure the precise integration of the targeting fragment at targeted allele, long-template PCR was next performed to amplify PCR products from the upstream of 5′ insertion site to the neo cassette (4.5 kb) and the downstream of 3′ insertion site to the neo cassette (6.0 kb) (Fig. 1*B*). Mating of heterozygous mice produced progeny of all three genotypes. Southern blots of *Eco*RI-digested tail DNA are shown in Fig. 1*C*, illustrating the predicted-sized fragments in *Ghsr*+/+, +/−, and −/− mice. Deletion of the *Ghsr* was also confirmed by the lack of hybridization to a probe selective for *Ghsr*-coding exon 1. Confirmation of the genotype was also provided by RT-PCR analysis of RNA isolated from mouse pituitary glands using oligonucleotide primers selected to prime in *Ghsr* coding exons 1 and 2. Fig. 1*D* illustrates the predicted-sized RT-PCR product (326 bp) in mRNA isolated from *Ghsr*+/+, but not in *Ghsr* −/− mice.

The well characterized properties of acute ghrelin administration are its stimulatory effects on GH release, appetite, and fat deposition (11, 14, 17). Therefore, if the GHSR is the biologically relevant ghrelin receptor, we might anticipate that the *Ghsr*-null mice would exhibit an anorexic dwarf phenotype. However, the appearance of *Ghsr*-null mice cannot be distinguished from that of their wild-type littermates. RT-PCR analysis indicated that ghrelin is expressed broadly in peripheral tissues (18); however, total necropsy of null- and wild-type mice and evaluation of hematoxylin/eosin-stained paraffin sections of individual tissues show no significant differences between the two genotypes. It has also been proposed that ghrelin plays a role in testicular (19) and placental function (20), but breeding of *Ghsr* heterozygous mice produced normal size litters with normal Mendelian distribution in genotype and sex. Furthermore, the homozygous litters produced by null parents showed no difference in body weight compared to wild-type litters of wild-type parents at birth and postnatal days (Fig. 1*E*). Collectively, these observations suggest that, if the GHSR is the ghrelin receptor, the physiological role of ghrelin is subtle.

Acute administration of ghrelin to wild-type animals stimulates GH release (11). To test whether ghrelin's effect on GH was mediated by the GHSR, we compared the effects of exogenous ghrelin in wild-type and *Ghsr*-null mice. Serum GH levels were measured in each mouse before ghrelin treatment, and at 5 and 15 min after treatment with vehicle or ghrelin. It is clear from the results in Fig. 2*A* that, in contrast to the response in wild-type mice, ghrelin fails to stimulate GH release in *Ghsr*-null mice, which shows unambiguously that the stimulatory effect of ghrelin on GH release is mediated by the GHSR. The GH-stimulatory effect of MK-0677 was also tested. Similar to that of ghrelin, GH release was only detected in wild-type mice, but not in *Ghsr*-null mice (Fig. 2*B*); therefore, the biological effects of ghrelin and MK-0677 on GH release are mediated by the GHSR.

Both GHSR agonists and GHRH stimulate GH release, but it is unknown whether these two signal pathways are dependent or independent. To test whether the GHRH–GH pathway remains functional in the absence of the *Ghsr*, we tested the stimulatory effect of GHRH in *Ghsr*-null mice. In contrast to treatment with ghrelin and MK-0677, GHRH stimulated GH release in both wild-type and null mice (Fig. 2*C*). These data show that the activity of GHRH does not depend on *Ghsr* expression.

Another well characterized property of ghrelin is its acute stimulatory effect on appetite (14, 17). To investigate whether the GHSR is indeed the ghrelin receptor that controls appetite, we compared food intake after ghrelin administration. Fig. 2*D* illustrated food intake in *Ghsr*-null and wild-type littermates that were treated in parallel with vehicle or 10 μg of ghrelin. We selected a dose of 10 μg per mouse because this dose produced serum ghrelin levels in the range observed in fasted mice (data not shown). Food intake was measured 30 min and 60 min after the first ghrelin

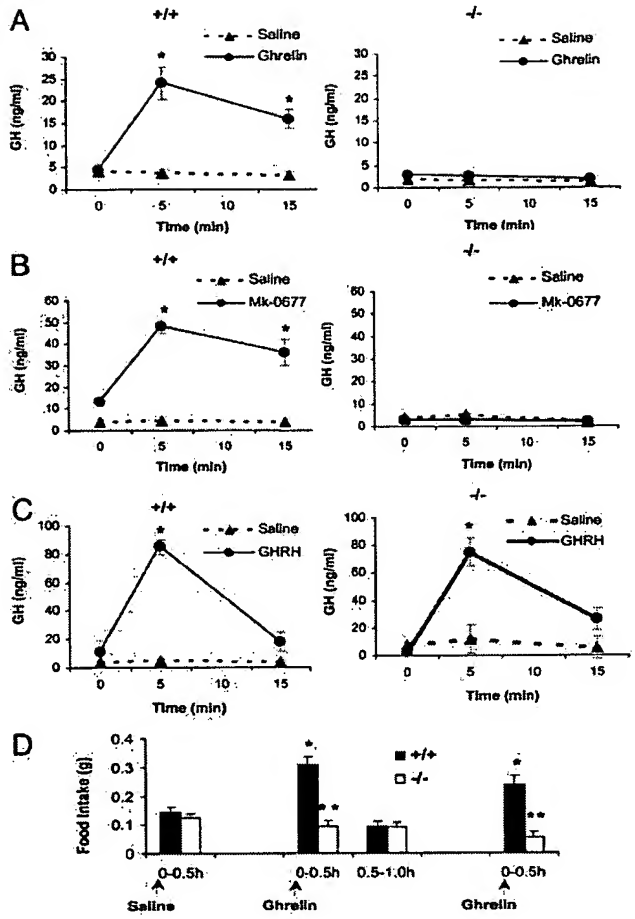


Fig. 2. (*A*) The effect of ghrelin administration on GH-release in *Ghsr*+/+ and *Ghsr*−/− mice ($n = 10$ for ghrelin and $n = 4$ for physiologic saline). At 5 min and 15 min after ghrelin injection, an asterisk shows $P < 0.001$ saline vs. ghrelin in +/+, but $P > 0.05$ in −/−. At 5 min and 15 min, $P < 0.001$ ghrelin-injected +/+ vs. −/−. (*B*) The effect of MK-0677 administration on GH-release in *Ghsr*+/+ and *Ghsr*−/− mice ($n = 5$ for MK-0677 and $n = 3$ for physiologic saline). At 5 min and 15 min after MK-0677 injection, an asterisk shows $P < 0.001$ saline vs. MK-0677 in +/+, but $P > 0.05$ in −/−. At 5 min and 15 min, $P < 0.001$ MK-0677-injected +/+ vs. −/−. (*C*) The effect of hGHRH administration on GH-release in *Ghsr*+/+ and *Ghsr*−/− mice ($n = 3$ for both hGHRH and physiologic saline). At 5 min after hGHRH injection, an asterisk shows $P < 0.001$ saline vs. hGHRH in both +/+ and −/−. GH release was stimulated in both +/+ and −/−. (*D*) Effects of ghrelin administration on food intake in *Ghsr*+/+ and *Ghsr*−/− mice. Mice were injected i.p. with 100 μl of physiologic saline first and food intake was measured at 0.5 h after the saline injection (0–0.5 h). Later, the same mice were injected with 100 μl of physiologic saline containing 10 μg of ghrelin (arrow in the middle). Food intake was measured at 0.5 h and 1.0 h after the ghrelin injection to get the food intake of the first 0.5 h (0–0.5 h) and the second 0.5h (0.5–1.0 h). One hour after the first ghrelin injection, ghrelin was reinjected (arrow on the right) and the food intake of the first 0.5 h (0–0.5 h) was remeasured. Food intake was significantly increased in the first 0.5 h after each ghrelin injection in +/+ (*, $P <$ 0.001 ghrelin vs. saline). There were no changes in food intake in −/− after ghrelin injection. For the second 0.5 h (0.5–1.0 h) of ghrelin injection, $P > 0.05$ saline vs. ghrelin for both +/+ and −/−. Arrows show the saline or ghrelin injections. $n = 10$. Double asterisk indicates $P < 0.001$ comparing +/+ vs. −/− during the first 0.5 h after each ghrelin injection. The same experiment was repeated three times on different days between 9 a.m. and 12 a.m. under ad libitum condition ($n = 10$ in each experiment).

injection. In wild-type mice, the 30-min food intake was unchanged after i.p. saline (0–0.5 h), but increased during the 30 min (0–0.5 h) immediately after i.p. ghrelin treatment ($P < 0.001$). During the second 30 min (0.5–1 h), food intake returned to control levels, which reflects the short half-life of ghrelin. After a second injection

of ghrelin, feeding was again stimulated. The duration and level of response to this dose of ghrelin was similar to that reported previously (17). In contrast to wild-type mice, ghrelin treatment did not influence food intake in *Ghsr*-null mice. These results were confirmed by experiments repeated 24 h later, and then 7 days later. Hence, stimulation of appetite by ghrelin is reproducible and depends on expression of the *Ghsr*.

Having established that the GHSR is the ghrelin receptor involved in the regulation of GH release and appetite, we investigated the metabolic characteristics of the *Ghsr*-null mice. It has been reported that reciprocal relationships exist between ghrelin and leptin and between ghrelin and insulin, during feeding and fasting (21, 22). Therefore, we measured the effects of fasting on ghrelin, leptin, and insulin levels in *Ghsr*-null mice and wild-type littermates. Remarkably, a similar increase of ghrelin was observed in both genotypes during fasting (Fig. 3*A*), illustrating that serum levels of ghrelin are not regulated by the GHSR. Fig. 3 *B* and *C* illustrates that fasting causes a parallel decline in leptin and insulin levels in both wild-type and null littermates, which suggests that ghrelin does not regulate leptin and insulin concentrations via the GHSR in both fed and fasted states.

Ghrelin is suggested to function as an antagonist of leptin on hypothalamic neurons (23), and because leptin action on the hypothalamus is reported to reduce bone density in rodents (24), we investigated whether *Ghsr*-null mice might exhibit reduced bone density. Fig. 3*D* shows that both bone mineral density and bone mass are comparable in *Ghsr* wild-type and null littermates, suggesting that the lack of a ghrelin receptor does not compromise significant bone growth. To more definitively evaluate the potential effects of ghrelin on bone during aging, comprehensive histological and morphological analysis will be carried out in isogenic strains of *Ghsr*-null mice.

A link between ghrelin and obesity has been made through the observations that, in obese humans who underwent gastric bypass surgery, ghrelin production declined in parallel with sustained weight loss and reduced appetite (25, 26). There is also a conflicting report that bypass surgery has no effect on ghrelin levels; the weight loss appeared to be obtained independently by the surgery (27). Recent studies show that ghrelin binds to terminals of neuropeptide Y (NPY)/Agouti-related protein (AGRP) neurons, and that a population of hypothalamic ghrelin-synthesizing neurons project to these terminals and modulate γ-aminobutyric acid (GABA) currents that are involved in appetite stimulation and corticotropin-releasing factor (CRF) release (28). To address whether a ghrelin/GHSR interaction is related to obesity, growth curves and food intake of *Ghsr*-null mice were monitored. The body weights of null mice were modestly lower than that of wild-type mice ($P < 0.05$) from 16 to 24 weeks of age (Fig. 4*A*). Although differences in body weight did not reach significance until the mice were 16 weeks old, the trend was present in younger animals. There was no significant difference in cumulative food intake (Fig. 4*B*) or biweekly food intake (data not shown) in 16- to 24-week-old *Ghsr*−/− mice compared to their wild-type littermates.

Ghrelin administration causes an acute increase in appetite, and serum ghrelin is up-regulated during fasting (17, 22), suggesting that ghrelin might be involved in fasting-induced hyperphagia. Interestingly, our data (Fig. 3*A*) showed that fasting increased serum ghrelin levels in *Ghsr* −/− mice as well. To further evaluate whether ghrelin is involved in reflex hyperphagia, we fasted the mice for 24 h, then refed them. The changes in body weight and food intake were identical in wild-type and *Ghsr*−/− littermates (Fig. 4 *C* and *D*). We also observed that there was no significant difference in short period (0.5 h, 1.0 h, and 2.0 h) food consumption after either 24 h or 48 h of fasting (data not shown). Our data show that the absence of the *Ghsr* has no effect on appetite, suggesting that ghrelin is not an essential orexigenic factor.

The reduced body weights of the *Ghsr*-null mice in Fig. 4*A* were not explained by either reduced bone density (Fig. 3*D*) or reduced food intake (Fig. 4*B*). Ghrelin has been suggested to be involved in fat utilization and deposition (17, 29), so we questioned whether the body composition of *Ghsr*-null mice is different from that of wild-type mice. Fig. 5*A* shows that, by peripheral instantaneous x-ray imager (PIXI) densitometry, there was no significant difference in fat ratio between the two genotypes. Fat and muscle mass were also determined by using a Minispec mq benchtop NMR spectrometer (Bruker Instruments). Although both fat and muscle content were found to be slightly less in *Ghsr*-null mice than in wild-type mice, these differences were not statistically significant (Table 1). In summary, our NMR data provided no clear explanation for the modestly lower weight exhibited by the *Ghsr*-null mice.

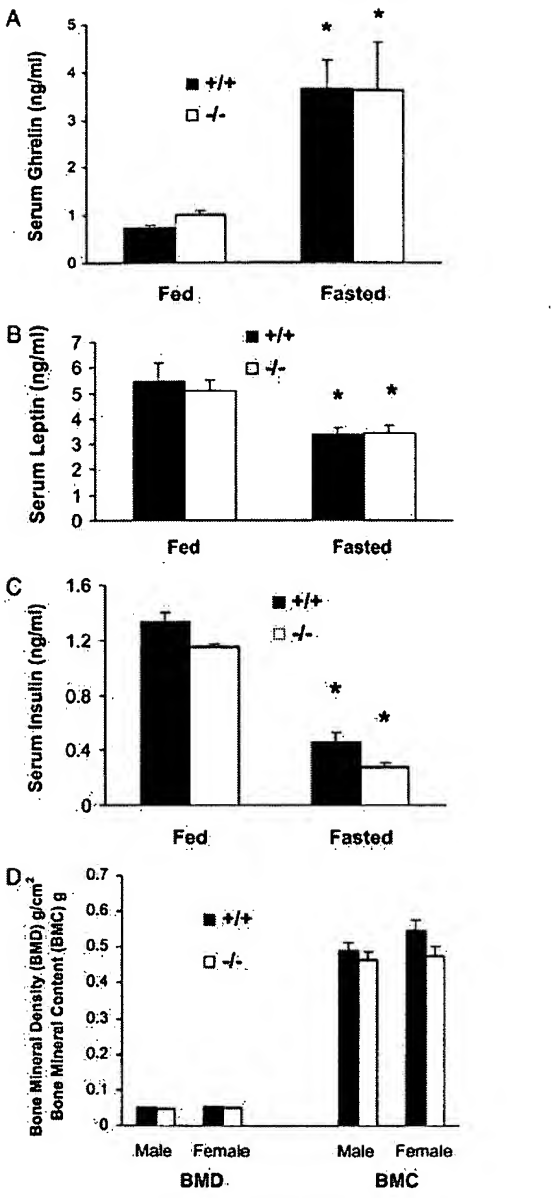


Fig. 3. (*A*) Fed and fasted serum ghrelin in 24-week-old male mice ($n = 7$, asterisk indicates $P < 0.05$ fed vs. fasted; $P > 0.05$ +/+ vs. −/− in both fed and fasted states). (*B* and *C*) Fed and fasted serum leptin and insulin of 20-week-old male mice ($n = 7$, asterisk indicates $P < 0.05$ fed vs. fasted for both leptin and insulin; $P > 0.05$ +/+ vs. −/− in both fed and fasted states). (*D*) Bone density and content of 24-week-old mice ($n = 7$, $P > 0.05$ +/+ vs. −/−). BMD, bone mineral density; BMC, bone mineral content.

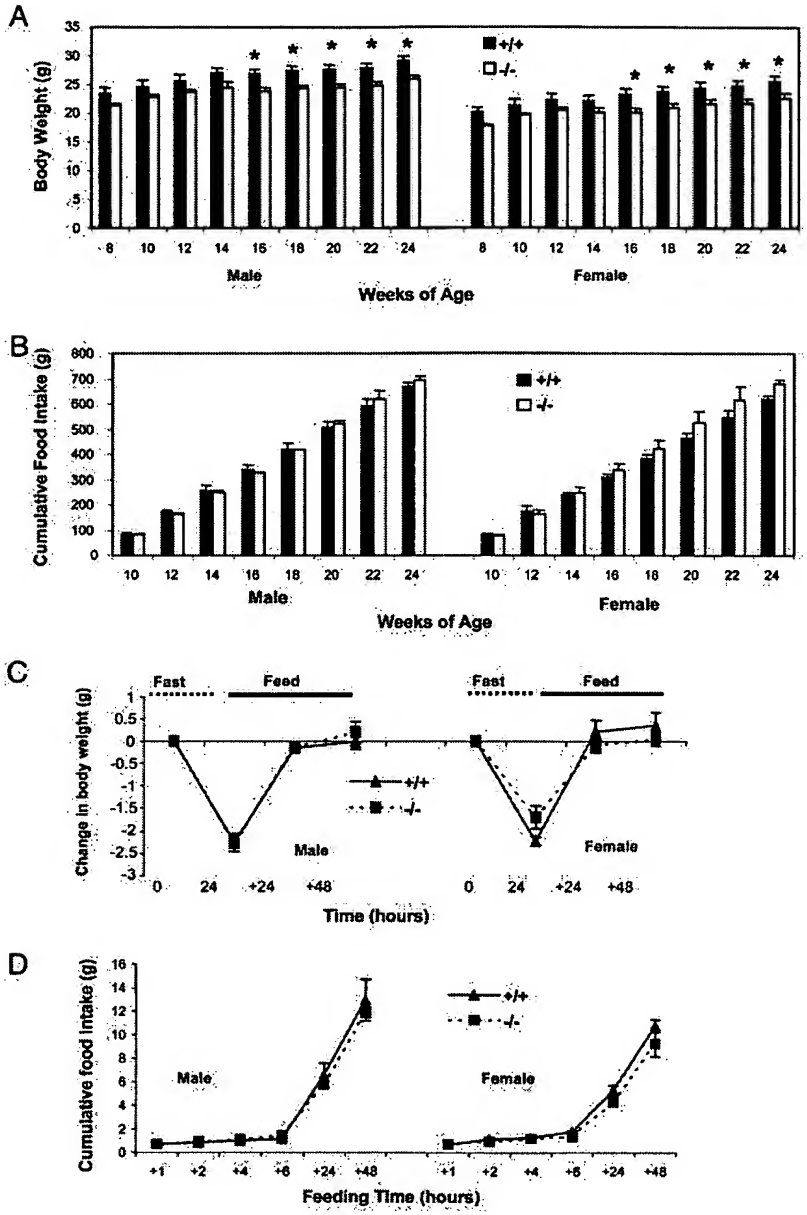


Fig. 4. (*A*) Body weights of 8- to 24-week-old mice. The data were collected every other week ($n = 7$, asterisk indicates $P < 0.05$ +/+ vs. −/− at 16–24 weeks). (*B*) Cumulative food intakes from 8 to 24 weeks of age ($n = 7$, $P > 0.05$ +/+ vs. −/− at all data points). (*C* and *D*) Changes in body weight and food intake during fasting and refeeding. Twelve-week-old mice were fasted for 24 h and then allowed to eat. Body weight was measured before and after the fasting, and at 24 h and 48 h after the food was given. Cumulative food intake was measured at 1, 2, 4, 6, 24, and 48 h after food was given ($n = 6$, $P > 0.05$ +/+ vs. −/−).

Because GHSR positively regulates levels of GH and IGF-1 (1), we predicted that the levels of these anabolic hormones would be lower in *Ghsr*-null mice. If GH and IGF-1 were lower, muscle mass and bone mass would be reduced; consequently, the modest reduction in body weights might be explained by subtle alterations in body composition caused by lower GH and IGF-1. The physiological profile of GH release is pulsatile; to make comparisons of the amplitude of GH pulses, sequential blood samples should be collected from a conscious animal at 10-min intervals for at least 12 h, which, to our knowledge, has never been accomplished in the mouse. However, serum IGF-1 does not exhibit pulsatility, and under conditions of similar nutritional status, reflects the basal GH profile. A comparison of IGF-1 levels showed that indeed IGF-1 was lower in the *Ghsr*-null mice (Fig. 5*B*, $P < 0.05$). Consequently, we speculate that the modestly lower body weight exhibited by the *Ghsr*-null mice is explained by subtle reductions in both muscle and bone mass, which when measured individually do not reach statistical significance.

It has been reported that long-lived Ames dwarf mice, which have reduced IGF-1 levels and are deficient in GH, prolactin, and thyroid stimulating hormone, have lower body temperature (30). We tested the rectal temperature of fed and 24- and 48-h fasted mice by using a temperature monitoring system from Indus Instruments (Houston, TX) and found no difference in core body temperature between null and wild-type mice, which suggests that, in contrast to the dwarf mice, the metabolic rate of the *Ghsr*-null mice is normal.

Our results with the *Ghsr*-null mice are consistent with earlier observations with long-acting ghrelin mimetics (1), but challenge

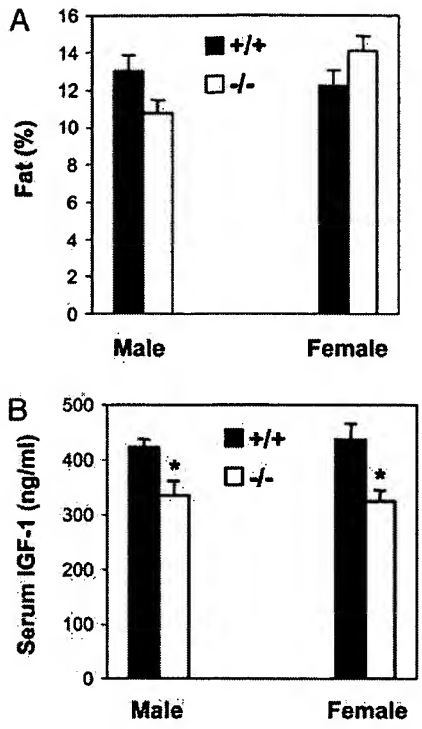


Fig. 5. Body composition (fat %) and serum IGF-1 of the mice in Fig. 4 *A* and *B* at 24 weeks of age. (*A*) Fat %, $n = 7$, $P > 0.05$ +/+ vs. −/−. (*B*) Serum IGF-1, $n = 7$, an asterisk indicates $P < 0.05$ +/+ vs. −/−.

the popular belief that ghrelin receptor null mice would have an anorexic dwarf phenotype. The anabolic effects of chronically stimulating this pathway were illustrated by increases in lean, but not fat, mass in obese subjects (7) and by the beneficial effects observed in treatment of a catabolic state (31). During aging, when ghrelin levels fall, the amplitude of GH pulsatility declines and serum IGF-1 levels drop (32). Restoration of depleted ghrelin levels would require either constant infusion of ghrelin or chronic treatment with a long-acting ghrelin mimetic. Indeed, chronic treatment of old animals with a ghrelin mimetic restores the physiology of the

Table 1. Body composition analysis of *Ghsr*-null mice by NMR spectroscopy

	Body weight	Fat	Muscle	Free fluid
+/+				
Mean, g	31.33	4.35	22.54	0.52
SEM, g	1.2	0.59	0.61	0.05
−/−				
Mean, g	28.14	3.33	21.29	0.71
SEM, g	0.54	0.46	0.46	0.1
P, $n = 7$	0.0451	0.1837	0.1209	0.1995

GH/IGF-1 axis to that of young adults (6). The observations that IGF-1 levels were lower and body weight was modestly reduced in *Ghsr*-null mice supports our early hypothesis that the GHSR is an enhancer of function (1), and is consistent with observations that ghrelin mimetics produce a sustained increase in the electrophysiological activity of hypothalamic arcuate neurons (33). We speculate that ghrelin enhances function of the GH/IGF-1 axis by modulating the "gain" or "set-point" of GHRH neurons.

The results of experiments in *Ghsr*-null mice show unambiguously that the GHSR is the physiologically relevant receptor controlling ghrelin's stimulatory effects on GH secretion and appetite. Because the appearance of *Ghsr*-null and wild-type mice is similar, it is unlikely that ghrelin plays a dominant role in determining growth and body composition. This conclusion is subject to the caveat that alternative pathways might compensate for the inability of the *Ghsr*-null mice to respond to ghrelin. Nevertheless, it seems unlikely that regulation of growth and appetite would be subject to equivalent compensation, and that ghrelin antagonists would be broadly efficacious antiobesity agents.

We thank Dr. Kevin Behar at Yale Mouse Metabolic Phenotyping Center for NMR body composition analysis (supported by National Institute of Diabetes and Digestive Kidney Diseases Grant U24 DK 59635) and Merck Research Laboratories for providing MK-0677. We thank Dr. Mark Asnicar for his valuable input, Adelina Gunawan for excellent technical assistance, Michael R. Honig for proofreading the manuscript, and Edith A. Gibson for preparing and editing the manuscript. We gratefully acknowledge the support of the National Institutes of Aging (Grants RO1AG18895 and RO1AG19230), the Hankamer Foundation, and the postdoctoral fellowship for Y.S. from Canadian Institutes of Health Research.

1. Smith, R. G., Van der Ploeg, L. H., Howard, A. D., Feighner, S. D., Cheng, K., Hickey, G. J., Wyvratt, M. J., Jr., Fisher, M. H., Nargund, R. P. & Patchett, A. A. (1997) *Endocr. Rev.* **18**, 621–645.
2. Momany, F. A., Bowers, C. Y., Reynolds, G. A., Chang, D., Hong, A. & Newlander, K. (1981) *Endocrinology* **108**, 31–39.
3. Smith, R. G., Cheng, K., Schoen, W. R., Pong, S.-S., Hickey, G. J., Jacks, T. M., Butler, B. S., Chan, W. W.-S., Chaung, L.-Y. P., Judith, F., *et al.* (1993) *Science* **260**, 1640–1643.
4. Smith, R. G., Pong, S.-S., Hickey, G. J., Jacks, T. M., Cheng, K., Leonard, R. J., Cohen, C. J., Arena, J. P., Chang, C. H., Drisko, J. E., *et al.* (1996) *Rec. Prog. Horm. Res.* **51**, 261–286.
5. Patchett, A. A., Nargund, R. P., Tata, J. R., Chen, M.-H., Barakat, K. J., Johnston, D. B. R., Cheng, K., Chan, W. W.-S., Butler, B. S., Hickey, G. J., *et al.* (1995) *Proc. Natl. Acad. Sci. USA* **92**, 7001–7005.
6. Chapman, I. M., Bach, M. A., Van, C. E., Farmer, M., Krupa, D. A., Taylor, A. M., Schilling, L. M., Cole, K. Y., Skiles, E. H., Pezzoli, S. S., *et al.* (1996) *J. Clin. Endocrinol. Metab.* **81**, 4249–4257.
7. Svensson, J., Lonn, L., Jansson, J.-O., Murphy, G., Wyss, D., Krupa, D., Cerchio, K., Polvino, W., Gertz, B., Boseaus, I., *et al.* (1998) *J. Clin. Endocrinol. Metab.* **83**, 362–369.
8. Howard, A. D., Feighner, S. D., Cully, D. F., Arena, J. P., Liberator, P. A., Rosenblum, C. I., Hamelin, M., Hreniuk, D. L., Palyha, O. C., Anderson, J., *et al.* (1996) *Science* **273**, 974–977.
9. Guan, X. M., Yu, H., Palyha, O. C., McKee, K. K., Feighner, S. D., Sirinathsinghji, D. J., Smith, R. G., Van der Ploeg, L. H. & Howard, A. D. (1997) *Brain Res. Mol. Brain Res.* **48**, 23–29.
10. Smith, R. G., Feighner, S., Prendergast, K., Guan, X. & Howard, A. (1999) *Trends Endocrinol. Metab.* **10**, 128–135.
11. Kojima, M., Hosoda, H., Date, Y., Nakazato, M., Matsuo, H. & Kangawa, K. (1999) *Nature* **402**, 656–660.
12. Tullin, S., Hansen, B. S., Ankersen, M., Moller, J., Von Cappelen, K. A. & Thim, L. (2000) *Endocrinology* **141**, 3397–3402.
13. Smith, R. G., Griffin, P. R., Xu, Y., Smith, A. G., Liu, K., Calacay, J., Feighner, S. D., Pong, C., Leong, D., Pomes, A., *et al.* (2000) *Biochem. Biophys. Res. Commun.* **276**, 1306–1313.
14. Nakazato, M., Murakami, N., Date, Y., Kojima, M., Matsuo, H., Kangawa, K. & Matsukura, S. (2001) *Nature* **409**, 194–198.
15. Papotti, M., Ghe, C., Cassoni, P., Catapano, F., Deghenghi, R., Ghigo, E. & Muccioli, G. (2000) *J. Clin. Endocrinol. Metab.* **85**, 3803–3807.
16. Silva Elipe, M. V., Bednarek, M. A. & Gao, Y. D. (2001) *Biopolymers* **59**, 489–501.
17. Wren, A. M., Small, C. J., Abbott, C. R., Dhillo, W. S., Seal, L. J., Cohen, M. A., Batterham, R. L., Taheri, S., Stanley, S. A., Ghatei, M. A., *et al.* (2001) *Diabetes* **50**, 2540–2547.
18. Gnanapavan, S., Kola, B., Bustin, S. A., Morris, D. G., McGee, P., Fairclough, P., Bhattacharya, S., Carpenter, R., Grossman, A. B. & Korbonits, M. (2002) *J. Clin. Endocrinol. Metab.* **87**, 2988.
19. Tena-Sempere, M., Barreiro, M. L., Gonzalez, L. C., Gaytan, F., Zhang, F. P., Caminos, J. E., Pinilla, L., Casanueva, F. F., Dieguez, C. & Aguilar, E. (2002) *Endocrinology* **143**, 717–725.
20. Gualillo, O., Caminos, J., Blanco, M., Garcia-Caballero, T., Kojima, M., Kangawa, K., Dieguez, C. & Casanueva, F. (2001) *Endocrinology* **142**, 788–794.
21. Bagnasco, M., Kalra, P. S. & Kalra, S. P. (2002) *Endocrinology* **143**, 726–729.
22. Toshinai, K., Mondal, M. S., Nakazato, M., Date, Y., Murakami, N., Kojima, M., Kangawa, K. & Matsukura, S. (2001) *Biochem. Biophys. Res. Commun.* **281**, 1220–1225.
23. Traebert, M., Riediger, T., Whitebread, S., Scharrer, E. & Schmid, H. A. (2002) *J. Neuroendocrinol.* **14**, 580–586.
24. Ducy, P., Amling, M., Takeda, S., Priemel, M., Schilling, A. F., Beil, F. T., Shen, J., Vinson, C., Rueger, J. M. & Karsenty, G. (2000) *Cell* **100**, 197–207.
25. Cummings, D. E., Weigle, D. S., Frayo, R. S., Breen, P. A., Ma, M. K., Dellinger, E. P. & Purnell, J. Q. (2002) *N. Engl. J. Med.* **346**, 1623–1630.
26. Leonetti, F., Silecchia, G., Iacobellis, G., Ribaudo, M. C., Zappaterreno, A., Tiberti, C., Iannucci, C. V., Perrotta, N., Bacci, V., Basso, M. S., *et al.* (2003) *J. Clin. Endocrinol. Metab.* **88**, 4227–4231.
27. Holdstock, C., Engstrom, B. E., Ohrvall, M., Lind, L., Sundbom, M. & Karlsson, F. A. (2003) *J. Clin. Endocrinol. Metab.* **88**, 3177–3183.
28. Cowley, M. A., Smith, R. G., Diano, S., Tschop, M., Pronchuk, N., Grove, K. L., Strasburger, C. J., Bidlingmaier, M., Esterman, M., Heiman, M. L., *et al.* (2003) *Neuron* **37**, 649–661.
29. Tschop, M., Smiley, D. L. & Heiman, M. L. (2000) *Nature* **407**, 908–913.
30. Hunter, W. S., Croson, W. B., Bartke, A., Gentry, M. V. & Meliska, C. J. (1999) *Physiol. Behav.* **67**, 433–437.
31. Murphy, M. G., Plunkett, L. M., Gertz, B. J., He, W., Wittreich, J., Polvino, W. M. & Clemmons, D. R. (1998) *J. Clin. Endocrinol. Metab.* **83**, 320–325.
32. Rigamonti, A. E., Pincelli, A. I., Corra, B., Viarengo, R., Bonomo, S. M., Galimberti, D., Scacchi, M., Scarpini, E., Cavagnini, F. & Muller, E. E. (2002) *J. Endocrinol.* **175**, R1–R5.
33. Bailey, A. R. T., Smith, R. G. & Leng, G. (1998) *J. Neuroendocrinol.* **10**, 111–118.

STOMACH

Antagonism of ghrelin receptor reduces food intake and body weight gain in mice

A Asakawa, A Inui, T Kaga, G Katsuura, M Fujimiya, M A Fujino, M Kasuga

Gut 2003;**52**:947–952

See end of article for authors' affiliations

Correspondence to: Dr A Inui, Division of Diabetes, Digestive and Kidney Diseases, Department of Clinical Molecular Medicine, Kobe University Graduate School of Medicine, 7-5-2 Kusunoki-cho, Chuo-ku, Kobe 650–0017, Japan; inui@med.kobe-u.ac.jp

Accepted for publication 8 January 2003

Background and aims: Ghrelin, an endogenous ligand for growth hormone secretagogue receptor (GHS-R), is an appetite stimulatory signal from the stomach with structural resemblance to motilin. We examined the effects of the gastric peptide ghrelin and GHS-R antagonists on energy balance and glycaemic control in mice.

Materials and methods: Body weight, fat mass, glucose, insulin, and gene expression of leptin, adiponectin, and resistin in white adipose tissue (WAT) were measured after repeated administrations of ghrelin under a high fat diet. Gastric ghrelin gene expression was assessed by northern blot analysis. Energy intake and gastric emptying were measured after administration of GHS-R antagonists. Repeated administration of GHS-R antagonist was continued for six days in *ob/ob* obese mice.

Results: Ghrelin induced remarkable adiposity and worsened glycaemic control under a high fat diet. Pair feeding inhibited this effect. Ghrelin elevated leptin mRNA expression and reduced resistin mRNA expression. Gastric ghrelin mRNA expression during fasting was increased by a high fat diet. GHS-R antagonists decreased energy intake in lean mice, in mice with diet induced obesity, and in *ob/ob* obese mice; it also reduced the rate of gastric emptying. Repeated administration of GHS-R antagonist decreased body weight gain and improved glycaemic control in *ob/ob* obese mice.

Conclusions: Ghrelin appears to be closely related to excess weight gain, adiposity, and insulin resistance, particularly under a high fat diet and in the dynamic stage. Gastric peptide ghrelin and GHS-R may be promising therapeutic targets not only for anorexia-cachexia but also for obesity and type 2 diabetes, which are becoming increasingly prevalent worldwide.

Ghrelin, a 28 amino acid peptide with an n-octanoyl modification on Ser 3, was recently identified in the stomach as an endogenous ligand for growth hormone secretagogue receptor (GHS-R).[1] The ghrelin gene is highly expressed in the stomach, and ghrelin circulates in human blood at a considerable plasma concentration.[1] This gut peptide with structural resemblance to motilin is involved in the regulation of growth hormone (GH) secretion, energy balance, gastric motility, and anxiety.[1–7] Previous studies have shown that administered ghrelin induces body weight gain and adiposity in rodents by promoting food intake and decreasing fat utilisation or energy expenditure.[2–5] Serum ghrelin concentrations are increased by fasting and are reduced by refeeding or oral and intravenous glucose administration.[8 9] In addition, ghrelin as well as synthetic growth hormone secretagogues, have been reported to stimulate feeding in humans.[2 6] These findings indicate that the gastric peptide ghrelin and GHS-R may be involved in the pathophysiology of obesity associated with an increased risk of diabetes, hypertension, hyperlipidaemia, osteoarthritis, and certain forms of cancer. Therefore, in the present study, we examined the role of ghrelin in diet induced obesity as well as the therapeutic potential of GHS-R antagonist in murine models of obesity.

METHODS

Animal experiments

Male mice of the ddy strain (34–37 g; Japan Slc, Shizuoka, Japan) and obese (*ob/ob*) C57BL/6J mice (68–74 g; Shionogi Co. Ltd, Shiga, Japan) were used. They were individually housed in a regulated environment (22±2°C, 55±10% humidity, 12:12 hour light:dark cycle with lights on at 7:00 am). Mice received a standard diet containing 12% of total energy as fat or a high fat diet containing 45% of total energy as fat (Clea Japan Inc., Tokyo, Japan). Food and water were available ad libitum except as otherwise indicated. All experiments were approved by our university animal care committee. [D-Lys-3]-GHRP-6, [D-Arg-1, D-Phe-5, D-Trp-7, 9, Leu-11] substance P, and rat ghrelin were purchased from Bachem California Inc. (Torrance, California, USA), Neosystem (Strasbourg, France), and Peptide Institute (Osaka, Japan), respectively. Just before administration, each drug was diluted in 4 µl of artificial cerebrospinal fluid for intra-third cerebroventricular (ICV) injection or in 100 µl of physiological saline for intraperitoneal injection. Doses of GHS-R antagonists were determined from our preliminary experiments on food intake and affinities for GHS- R.[10–12] Results are expressed as means (SEM). Analysis of variance (ANOVA) followed by Bonferroni's *t* test were used to assess differences among groups: $p<0.05$ was considered to be statistically significant.

ICV substance application

For ICV injection, mice were anaesthetised with sodium pentobarbital (80–85 mg/kg intraperitoneally) and placed in a stereotaxic instrument seven days before the experiments. A hole was made in each skull using a needle inserted 0.9 mm lateral to the central suture and 0.9 mm posterior to the bregma. A 24 gauge cannula bevelled at one end over a distance of 3 mm was implanted into the third cerebral ventricle for ICV injection. The cannula was fixed to the skull with dental cement and capped with silicon without an obtruder. A 27 gauge injection insert was attached to a microsyringe by PE-20 tubing.

Abbreviations: AGRP, agouti related protein; ARC, arcuate nucleus; FFA, free fatty acids; GH, growth hormone; GHRP, growth hormone releasing peptide; GHS-R, growth hormone secretagogue receptor; ICV, intra-third cerebroventricular(ly); NPY, neuropeptide Y; WAT, white adipose tissue.

Table 1 Effects of ghrelin administered intraperitoneally (3 nmol/mouse every 12 hours for five days) on calorie intake, epididymal fat mass, gastrocnemius muscle, and blood glucose, insulin, cholesterol, triglycerides, and free fatty acid concentrations in lean mice under a high fat diet

	LF, saline	HF, saline	HF, ghrelin
Calorie intake (kcal/day)	18.83 (1.055)	23.22 (1.329)	25.94 (2.562)*
Fat pad mass(g)	0.533 (0.049)	0.797 (0.095)	1.202 (0.175)*†
Skeletal musde (g)	0.337 (0.016)	0.353 (0.010)	0.340 (0.005)
Glucose (mmol/l)	7.899 (0.476)	8.393 (0.825)	8.909 (0.498)
Insulin (pmol/l)	135.0 (20.10)	177.5 (13.05)	378.0 (141.8)*
Cholesterol (mmol/l)	3.742 (0.337)	5.568 (0.497)	5.813 (0.509)**
Triglycerides (mmol/l)	0.347 (0.032)	0.305 (0.039)	0.371 (0.090)
Free fatty acids (meq/l)	1.467 (0.050)	1.623 (0.100)	1.636 (0.047)

Results are expressed as mean (SEM).
LF and HF indicate a standard diet and a high fat diet, respectively.
*p<0.05, **p<0.01 between saline treated mice fed a standard diet and ghrelin treated mice fed a high fat diet.
†p<0.05 between saline treated mice fed a high fat diet and ghrelin treated mice fed a high fat diet.

Feeding tests

Experiments were started at 10:00 am. Before feeding tests, mice were food deprived for 16 hours with free access to water, except for the experiment of the effect of coadministration of [D-Lys-3]-GHRP-6 and ghrelin on food intake, in which mice were given free access to food and water. Food intake was measured by subtracting uneaten food from the initially premeasured food at 20 minutes, one, two, and four hours after administration.

RNA isolation and northern blot analysis

RNA was isolated from the stomach and epididymal fat using the RNeasy Mini Kit (Qiagen, Tokyo, Japan). Total RNA was denatured with formaldehyde, electrophoresed in 1% agarose gel, and blotted onto a Hybond N+ membrane. The membranes were hybridised with a fluoresceine labelled cDNA probe. The total integrated densities of hybridisation signals were determined by densitometry (Amersham Pharmacia Biotech AB, Uppsala, Sweden). Data were normalised to glyceraldehyde 3-phosphate dehydrogenase mRNA abundance and expressed as a percentage of controls.

Ghrelin gene expression

Lean mice received a standard diet containing 12% of total energy as fat or a high fat diet containing 45% of total energy as fat for two weeks. Mice were fasted for eight hours before being killed by cervical dislocation. Immediately after, stomachs were removed, frozen on dry ice, and stored at −80°C until preparation of northern blots.

Gastric emptying

Before the gastric emptying experiments, mice were food deprived for 16 hours with free access to water. Fasted mice had free access to preweighed pellets for one hour and were then administered [D-Lys-3]-GHRP-6. Mice were deprived of food again for one or two hours after injection. Food intake was measured by weighing uneaten pellets. Mice were killed by cervical dislocation two or three hours after the start of the experiments. Immediately after the stomach was exposed by laparotomy, quickly ligated at both the pylorus and cardia, removed, and the dry content was weighed. Gastric emptying was calculated according to the following formula:

$$\text{gastric emptying (\%)} = (1 - (\text{dry weight of food recovered from the stomach}/\text{weight of food intake})) \times 100.$$

Anxiety tests

Anxiety was assessed in the standard elevated plus maze, 50 cm above the ground.[11] The four arms were 27 cm long and 6 cm wide. Two opposing arms were enclosed by walls 15 cm high (closed arms) while the other arms were devoid of walls (open arms). Each mouse was placed in the centre of the maze facing one of the enclosed arms 10 minutes after injection. The cumulative time spent in each arm and the number of entries into the open or closed arms were recorded during a five minute test session. The time spent in the open arms was expressed as a percentage of total entry time (100×open/open+closed) and the number of entries in the open arms was expressed as a percentage of the total number of entries (100×open/total entries).

Repeated injection

Repeated intraperitoneal injections were continued for five days in lean mice under a high fat diet or a standard diet, and for six days in lean and *ob/ob* obese mice under a standard diet, respectively. Mice were injected daily at 7:00 am and 19:00 pm. Food intake and body weight were measured daily. For pair feeding studies, a pair fed group experiment was initiated one day after the pair group to match food intake. Serum was separated from blood obtained from the orbital sinus under ether anaesthesia at the end of the experiment (eight hours after removal of food and the final intraperitoneal injection). Mice were killed by cervical dislocation. Immediately after, the epididymal fat pad mass assessed as white adipose tissue (WAT) and the gastrocnemius muscle were removed and weighed. Blood glucose was measured by the glucose oxidase method. Serum insulin and free fatty acids (FFA) were measured by enzyme immunoassay and an enzymatic method (Eiken Chemical Co., Ltd, Tokyo, Japan), respectively. Serum triglycerides and total cholesterol were measured by an enzymatic method (Wako Pure Chemical Industries, Ltd, Tokyo, Japan).

RESULTS

We first examined the effects of repeated administration of ghrelin on body weight gain and glycaemic control under a high fat diet. Intraperitoneal injection of ghrelin twice daily for five days tended to increase body weight compared with saline treated mice fed a high fat diet. Body weight of saline treated mice fed a standard diet, saline treated mice fed a high fat diet, and ghrelin treated mice fed a high fat diet increased by 0.44 (0.13), 0.60 (0.12), and 0.92 (0.18) g/day, respectively. There were significant differences in fat pad mass both between saline treated mice fed a standard diet and ghrelin treated mice fed a high fat diet, and also between saline treated mice fed a high fat diet and ghrelin treated mice fed a high fat diet (table 1). Skeletal muscle did not show an increase in weight. Serum cholesterol and insulin levels were also increased, accompanied by a moderate increase in blood glucose concentrations. We then assessed mRNA levels of leptin, adiponectin, and resistin in WAT. Repeated ghrelin injections reduced resistin mRNA expression in WAT (fig 1A). Gene expression of leptin and adiponectin in ghrelin treated

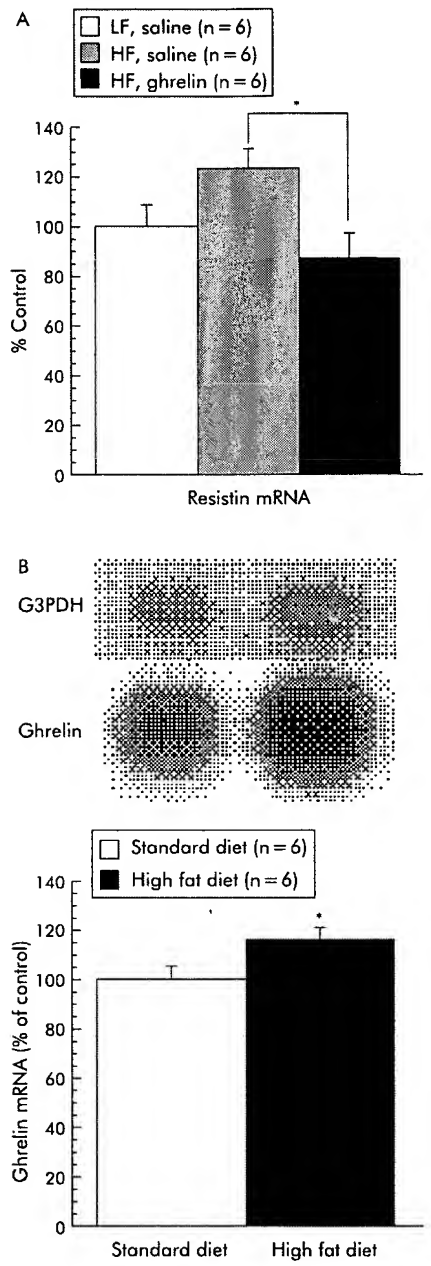


Figure 1 (A) Chronic effects of ghrelin administered intraperitoneally (3 nmol/mouse every 12 hours for five days) on resistin gene expression in the epididymal fat mass under a high fat (HF) diet, as assessed by northern blot analysis, expressed as a percentage of physiological saline treated controls under a standard (LF) diet. Results are expressed as mean (SEM): n indicates the number of mice used, *p<0.05 between saline treated mice fed a high fat diet and ghrelin treated mice fed a high fat diet. (B) Stimulatory effects of a high fat diet for two weeks on ghrelin gene expression in the stomach of food deprived mice, as assessed by northern blot analysis, expressed as a percentage of standard diet fed controls. (Top) A representative northern blot analysis showing gastric ghrelin mRNA during fasting after a high fat diet for two weeks; *p<0.05 compared with the control group by Bonferroni's *t* test. G3PDH, glyceraldehyde 3-phosphate dehydrogenase.

mice fed a high fat diet was increased by 71% and 3%, respectively, compared with saline treated mice fed a high fat diet; however, neither reached statistical significance. Comparison with pair fed controls confirmed that ghrelin had no significant effects on body weight gain (0.15 (0.09) (3 nmol) *v* 0.17 (0.09) g/day (control); n=5), fat pad mass (0.53 (0.02) *v* 0.51 (0.06) g (control) or blood glucose concentrations (6.82 (0.31) *v* 7.85 (0.29) mmol/l (control). Furthermore, we assessed ghrelin mRNA expression under a high fat diet containing 45% of total energy as fat. A high fat diet for two weeks significantly increased ghrelin gene expression in the stomach of food deprived mice compared with a standard diet (fig 1B). On the other hand, a high fat diet showed a tendency to decrease ghrelin gene expression in the fed state compared with a standard diet, although this effect failed to reach statistical significance (62.0 (8.53)% of control; n=5).

We determined whether GHS-R antagonists would influence energy balance. We first administered the GHS-R antagonist [D-Lys-3]-GHRP-6 IP to mice. As shown in fig 2A, [D-Lys-3]-GHRP-6 significantly decreased food intake in a dose related manner. We also investigated whether centrally administered [D-Lys-3]-GHRP-6 has similar effects. ICV as well as intraperitoneally administered [D-Lys-3]-GHRP-6 produced a potent decrease in feeding behaviour (fig 2B). To evaluate the possibility that ghrelin acts through GHS-R in the brain, we examined the effects of simultaneous administration of ghrelin and [D-Lys-3]- GHRP-6 on food intake. ICV administered [D-Lys-3]-GHRP-6 abolished the stimulatory effects on feeding induced by intraperitoneal injection of ghrelin (fig 2C). We next examined the effect of intraperitoneal injection of [D-Lys-3]-GHRP-6 on gastric emptying rate. Peripherally administered [D-Lys-3]-GHRP-6 produced a significant decrease in the gastric emptying rate one hour after administration (fig 2D). No gross behavioural changes were observed after [D-Lys-3]-GHRP-6 administration. In anxiety tests, there were no significant differences in the percentage of total time spent in the open arms (16.3 (10.1) (200 nmol) *v* 13.8 (4.92)% (control); n=5) and the total number of entries into the open arms (20.6 (7.18) *v* 16.7 (5.75)% (control)) between control and [D-Lys-3]-GHRP-6 treated animals. We also examined the effect of another GHS-R antagonist [D-Arg-1, D-Phe-5, D-Trp-7, 9, Leu- 11] substance P (L-756,867) on feeding in food deprived mice. Peripherally administered [D- Arg-1, D-Phe-5, D-Trp-7, 9, Leu-11] substance P as well as [D-Lys-3]-GHRP-6 significantly decreased food intake in a dose dependent manner (fig 2E). Moreover, we examined the effect of [D-Lys-3]-GHRP-6 in mice rendered obese by a high fat diet. Intraperitoneal administration of [D-Lys-3]-GHRP-6 potently decreased food intake, leading to decreased body weight gain (fig 2F). Seven daily administrations of [D-Lys-3]-GHRP-6 had a tendency to decrease body weight gain (0.18 (0.12) (200 nmol) *v* 0.39 (0.10) g/day (control); n=6), fat pad mass (1.37 (0.15) *v* 1.72 (0.23) g (control)), and blood glucose concentrations (8.85 (0.39) *v* 9.28 (0.74) mmol/l (control)) in mice rendered obese by the diet. In addition, comparison with pair fed controls indicated that [D-Lys-3]-GHRP-6 had no significant effects on body weight gain (0.09 (0.09) (200 nmol) *v* 0.08 (0.05) g/day (control); n=5), fat pad mass (0.49 (0.06) *v* 0.52 (0.07) g (control)) or blood glucose concentrations (7.73 (0.39) *v* 7.07 (0.41) mmol/l (control)) in lean mice.

To gain further insight into the therapeutic potential, we examined whether or not intraperitoneally administered [D-Lys-3]-GHRP-6 produced anorexigenic effects in *ob/ob* obese mice. [D-Lys-3]-GHRP-6 significantly decreased food intake in *ob/ob* obese mice as well as in lean mice (fig 3A). Finally, we examined the effects of repeated administration of [D-Lys-3]-GHRP-6 on body weight gain and glycaemic control in *ob/ob* obese mice. Repeated injections of [D-Lys-3]-GHRP-6 significantly lowered body weight gain and blood glucose concentrations without decreasing muscle weight (fig 3B, table 2). Furthermore, [D-Lys-3]-GHRP-6 treatment significantly reduced FFA levels of *ob/ob* obese mice by 24% compared with

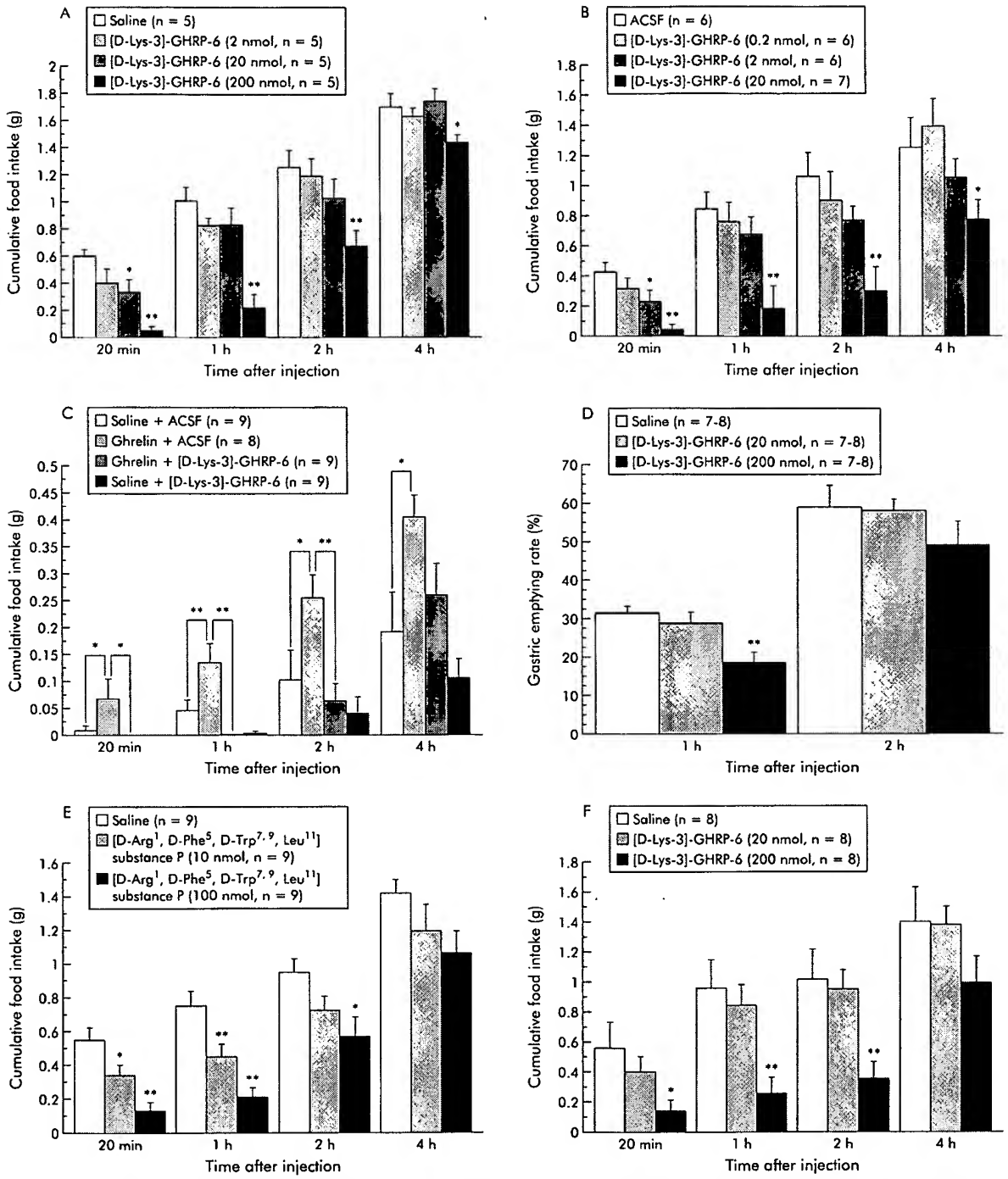


Figure 2 (A) Effects of intraperitoneally administered [D-Lys-3]-GHRP-6 (2–200 nmol/mouse) on cumulative food intake in food deprived lean mice: *p<0.05, **p<0.01 compared with the control group by Bonferroni's *t* test. (B) Effects of intracerebroventricularly administered [D-Lys-3]-GHRP-6 (0.2–20 nmol/mouse) on cumulative food intake in food deprived lean mice. ACSF, artificial cerebrospinal fluid. (C) Antagonistic effects of [D-Lys-3]-GHRP-6 administered intracerebroventricularly (20 nmol/mouse) on feeding induced by intraperitoneal injection of ghrelin (3 nmol/mouse) in non-food deprived lean mice. (D) Inhibitory effects of intraperitoneally administered [D-Lys- 3]-GHRP-6 (20–200 nmol/mouse) on the gastric emptying rate one and two hours after injection in lean mice. (E) Effects of intraperitoneally administered [D-Arg-1, D-Phe-5, D- Trp-7, 9, Leu-11] substance P (10–100 nmol/mouse) on cumulative food intake in food deprived lean mice. (F) Effects of intraperitoneally administered [D-Lys-3]-GHRP-6 (20–200 nmol/mouse) on cumulative food intake in mice with diet induced obesity who received a high fat diet for two weeks.

saline treated *ob/ob* obese mice (fig 3C). Control and [D-Lys-3]-GHRP-6 treated animals exhibited no significant differences in water intake (10.5 (0.43 (200 nmol) *v* 10.7 (0.43) ml/day (control); n=7).

DISCUSSION

The pathophysiology of obesity is known to be sustained excess of nutrient intake over expenditure. It has been shown that a "Western diet" with a high fat content is associated with

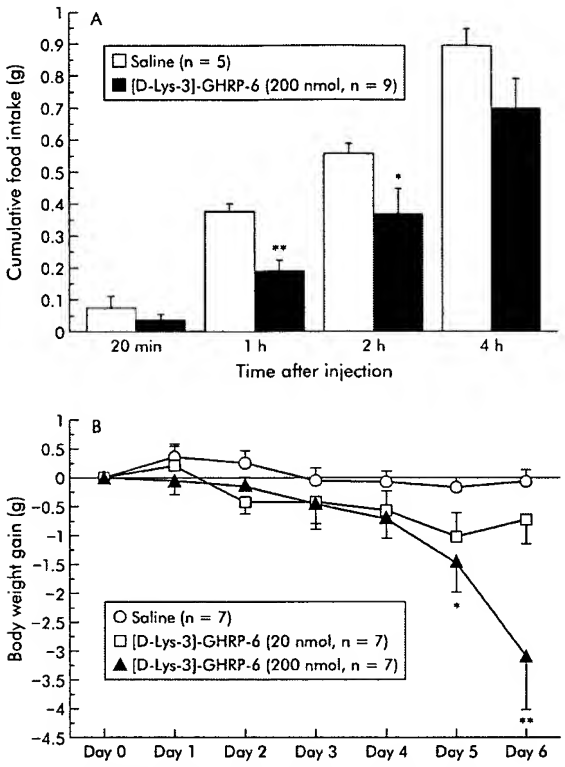


Figure 3 (A) Acute effects of intraperitoneally administered [D-Lys-3]-GHRP-6 (200 nmol/mouse) on cumulative food intake in food deprived *ob/ob* obese mice: *p<0.05, **p<0.01 compared with physiological saline treated controls. (B) Chronic effects of [D-Lys- 3]-GHRP-6 administered intraperitoneally (20–200 nmol/mouse every 12 hours for six days) on body weight gain in non-food deprived *ob/ob* obese mice.

an increased risk of obesity. We previously reported that repeated administration of ghrelin significantly increased body weight compared with saline treated controls under a standard diet.[4] Fat pad mass, serum insulin, and cholesterol levels showed a tendency to increase by 31.8%, 76.2%, and 12.9%, respectively, although they failed to reach statistical significance. In this study, we found that repeated administration of ghrelin significantly increased adiposity with a concomitant increase in cholesterol and insulin levels under a high fat diet.

Leptin, adiponectin, and resistin are known to be associated with insulin resistance as adipocytokines, in WAT. Leptin is thought to be a critical molecule in the body weight regulatory system as an adiposity signal from the periphery to the hypothalamus.[2 14–16] On the other hand, previous studies have shown that hypoadiponectinaemia is closely related to hyperinsulinaemia and insulin resistance, which is ameliorated by adiponectin treatment.[17] Resistin, a new adipose secreted polypeptide, has been reported to have a causative role in insulin resistance through an as yet unknown mechanism.[18] However, this was recently challenged by conflicting data on resistin gene expression. While tumour necrosis factor α and FFA, which contribute to insulin resistance, have a suppressive effect on resistin mRNA levels in adipocytes, several types of peroxisome proliferator activated receptor gamma agonists, antidiabetic drugs with an insulin sensitising action, increase adipose resistin mRNA expression.[19 20] In addition, although serum levels of resistin are increased in genetic and diet induced forms of obesity, resistin gene expression is reported to be suppressed by insulin and obesity.[18 21 22] In the present study, ghrelin elevated leptin mRNA expression, as well as reduced resistin mRNA expression in WAT with insulin resistance, which might be compatible with the latter postulate. Recently, Lee *et al* have reported that under ad libitum fed condition, gastric ghrelin gene expression was decreased by a high fat diet.[23] In contrast, we have shown here that under fasted condition, ghrelin gene expression in the stomach is increased by a high fat diet. These observations indicate that ghrelin appears to be closely related to excess weight gain, adiposity, and insulin resistance, particularly under a high fat diet and in the dynamic stage. If so, GHS-R may represent a target for pharmacological intervention in the treatment of obesity and related disorders.

Assuming that GHS-R antagonists would induce a state of negative energy balance, we examined the effects of GHS-R antagonists on feeding. As expected, GHS-R antagonists decreased feeding in lean mice and in mice rendered obese by a high fat diet. Previous reports have shown that GHS-R is present in the hypothalamus, heart, lung, pancreas, intestine, and adipose tissue.[1 2] In the hypothalamus, GHS-R is located in the arcuate nucleus (ARC), where two orexigenic peptides, neuropeptide Y (NPY) and agouti related protein (AGRP), are synthesised in the neurone.[1 2 16] In addition, non-peptide GH secretagogues act in the hypothalamus to alter the electrical activity of ARC neurones and activate expression of c- fos.[2 24] To date, ghrelin has been reported to stimulate feeding behaviour with its mechanism of action involving direct activation of hypothalamic NPY and AGRP neurones in the ARC where the blood-brain barrier is less effective.[2 4 5] However, an alternative pathway for ghrelin signalling from the stomach is via an ascending neural network through the vagus nerve and brainstem nuclei that ultimately reaches the hypothalamus.[2 4] In our study, centrally administered GHS-R antagonist abolished the stimulatory effects on feeding induced by peripherally

Table 2 Effects of [D-Lys-3]-GHRP-6 administered intraperitoneally (20–200 nmol/mouse every 12 hours for six days) on food intake, epididymal fat mass, gastrocnemius muscle, and blood glucose, insulin, cholesterol, triglyceride, and free fatty acid concentrations in *ob/ob* obese mice

	Saline	20 nmol	200 nmol
Food intake (g/day)	4.845 (0.160)	4.527 (0.261)	4.285 (0.298)
Fat pad mass (g)	0.974 (0.066)	0.897 (0.169)	0.860 (0.086)
Skeletal muscle (g)	0.300 (0.012)	0.314 (0.009)	0.326 (0.013)
Glucose (mmol/l)	13.01 (1.538)	12.06 (1.549)	7.489 (1.081)*
Insulin (pmol/1)	8294 (1676)	6242 (1628)	5481 (1304)
Cholesterol (mmol/1)	6.649 (0.346)	5.663 (0.291)	5.906 (0.565)
Triglycerides (mmol/l)	0.518 (0.049)	0.436 (0.040)	0.465 (0.068)
Free fatty acids (meq/l)	2.164 (0.075)	2.036 (0.121)	1.646 (0.078)**

*p<0.05, **p<0.01 compared with physiological saline treated controls.

administered ghrelin. These results suggest that ghrelin may act through GHS-R in the brain. We also demonstrated that peripherally administered GHS-R antagonist decreased gastric emptying rate which may contribute to its anorexigenic effect. Considerable evidence has accumulated to indicate that gastric distention acts as a satiety signal to inhibit food intake, and rapid gastric emptying is closely related to overeating and obesity, as is delayed gastric emptying to anorexia and cachexia.[25] Previous studies have shown that ghrelin increases gastric emptying rate and motility through vagal pathways.[2 4] In addition, ICV administered NPY affects gastroduodenal contractile activity, changing fed patterns of irregular contractions into phasic contractions characterised as fasted patterns.[26] Thus our findings suggest that GHS-R has a role in the control of feeding behaviour and that antagonism of GHS-R may be a promising approach for treating obesity.

Finally, we demonstrated that peripherally administered GHS-R antagonists produced anorexigenic effects and lowered body weight gain and blood glucose concentrations in *ob/ob* obese mice, which is a known genetic model of obesity and diabetes with insulin resistance and rapid gastric emptying.[16] This remarkable reduction in glucose levels, accompanied by a moderate decrease in serum insulin levels, implicates GHS-R antagonists in the amelioration of insulin resistance. In contrast, it has been shown that elevations of plasma FFA induce insulin resistance through inhibition of glucose transport activity with its mechanism of action involving reduction of phosphatidylinositol 3-kinase activity.[27] Recently, elevated circulating FFA concentration has been reported to be an independent risk factor for sudden death in middle aged men in a long term cohort study.[28] In the present study, GHS-R antagonists produced a remarkable decrease in FFA levels of *ob/ob* obese mice. In addition, GHS-R antagonists demonstrated no significant effects on water intake, general behaviour, or anxiety, compared with saline treated controls, suggesting the specific nature of the effect.

In conclusion, we found that peripherally administered GHS-R antagonists [D-Lys-3]- GHRP-6 and [D-Arg-1, D-Phe-5, D-Trp-7, 9, Leu-11] substance P decreased food intake in lean mice, in mice with diet induced obesity, and in *ob/ob* obese mice. We also showed that repeated administration of [D-Lys-3]-GHRP-6 decreased body weight gain and improved glycaemic control in *ob/ob* obese mice. In contrast, repeated administration of ghrelin, an endogenous ligand for GHS-R, induced remarkable adiposity and affected glycaemic control under a high fat diet. Moreover, gastric ghrelin gene expression during fasting was elevated by a high fat diet. Together with the findings that the stomach is not only a source of ghrelin but also a source of leptin,[29] the stomach as well as adipose tissue plays a crucial role in the regulation of energy balance as an endocrine organ. Hansen *et al* have recently reported that weight loss in obese human subjects increases fasting plasma ghrelin levels.[30] Moreover, transgenic rats expressing an antisense GHS-R mRNA have been reported to exhibit lower body weight and less adipose tissue than control rats.[31] Although further studies are needed to determine the possibility of GHS-R antagonists acting as toxins, these observations suggest that gastric peptide ghrelin and GHS-R may be promising targets for pharmacological intervention, not only in the treatment of anorexia-cachexia but also in the treatment of obesity and type 2 diabetes, which are increasingly prevalent in the world.

Authors' affiliations
A Asakawa, A Inui, T Kaga, M Kasuga, Division of Diabetes, Digestive and Kidney Diseases, Department of Clinical Molecular Medicine, Kobe University Graduate School of Medicine, Kobe 650-0017, Japan
G Katsuura, Shionogi Research Laboratories, Shionogi and Co Ltd, Shiga 520-3423, Japan
M Fujimiya, Department of Anatomy, Shiga University of Medical Science, Shiga 520-2192, Japan
M A Fujino, First Department of Internal Medicine, Yamanashi Medical University, Yamanashi 409-3898, Japan

REFERENCES

1 **Kojima M**, Hosoda H, Date Y, *et al.* Ghrelin is a growth-hormone-releasing acylated peptide from stomach. *Nature* 1999;**402**:656–60.
2 **Inui A.** Ghrelin: an orexigenic and somatotrophic signal from the stomach. *Nat Rev Neurosci* 2001;**2**:551–60.
3 **Tschop M**, Smiley DL, Heiman ML. Ghrelin induces adiposity in rodents. *Nature* 2000;**407**:908–13.
4 **Asakawa A**, Inui A, Kaga T, *et al.* Ghrelin is an appetite-stimulatory signal from stomach with structural resemblance to motilin. *Gastroenterology* 2001;**120**:337–45.
5 **Nakazato M**, Murakami N, Date Y, *et al.* A role for ghrelin in the central regulation of feeding. *Nature* 2001;**409**:194–8.
6 **Wren AM**, Seal LJ, Cohen MA, *et al.* Ghrelin enhances appetite and increases food intake in humans. *J Clin Endocrinol Metab* 2001;**86**:5992–5.
7 **Asakawa A**, Inui A, Kaga T, *et al.* A role of ghrelin in neuroendocrine and behavioral responses to stress in mice. *Neuroendocrinology* 2001;**74**:143–7.
8 **Cummings DE**, Purnell JQ, Frayo RS, *et al.* A preprandial rise in plasma ghrelin levels suggests a role in meal initiation in humans. *Diabetes* 2001;**50**:1714–19.
9 **Shiiya T**, Nakazato M, Mizuta M, *et al.* Plasma ghrelin levels in lean and obese humans and the effect of glucose on ghrelin secretion. *J Clin Endocrinol Metab* 2002;**87**:240–4.
10 **Cheng K**, Chan WW, Barreto A, *et al.* The synergistic effects of His-D-Trp-Ala-Trp- D-Phe-Lys-NH2 on growth hormone (GH)-releasing factor-stimulated GH release and intracellular adenosine 3', 5'-monophosphate accumulation in rat primary pituitary cell culture. *Endocrinology* 1989;**124**:2791–8.
11 **Veeraragavan K**, Sethumadhavan K, Bowers CY. Growth hormone-releasing peptide (GHRP) binding to porcine anterior pituitary and hypothalamic membranes. *Life Sci* 1992;**50**:1149–55.
12 **Cheng K**, Wei L, Chaung LY, *et al.* Inhibition of L-692,429-stimulated rat growth hormone release by a weak substance P antagonist: L-756,867. *J Endocrinol* 1997;**152**:155–8.
13 **Asakawa A**, Inui A, Momose K, *et al.* Endomorphins have orexigenic and anxiolytic activities in mice. *Neuroreport* 1998;**9**:2265–7.
14 **Flier JS**, Flier EM. Obesity and the hypothalamus: Novel peptides for new pathways. *Cell* 1998;**92**:437–40.
15 **Kalra SP**, Dube MG, Pu S, *et al.* Interacting appetite-regulating pathways in the hypothalamic regulation of body weight. *Endocr Rev* 1999;**20**:68–100.
16 **Inui A.** Transgenic approach to the study of body weight regulation. *Pharmacol Rev* 2000;**52**:35–61.
17 **Yamauchi T**, Kamon J, Waki H, *et al.* The fat-derived hormone adiponectin reverses insulin resistance associated with both lipoatrophy and obesity. *Nat Med* 2001;**7**:941–6.
18 **Steppan CM**, Bailey ST, Bhat S, *et al.* The hormone resistin links obesity to diabetes. *Nature* 2001;**409**:307–12.
19 **Fasshauer M**, Klein J, Neumann S, *et al.* Tumor necrosis factor alpha is a negative regulator of resistin gene expression and secretion in 3T3-L1 adipocytes. *Biochem Biophys Res Commun* 2001;**288**:1027–31.
20 **Juan CC**, Au LC, Fang VS, *et al.* Suppressed gene expression of adipocyte resistin in an insulin-resistant rat model probably by elevated free fatty acids. *Biochem Biophys Res Commun* 2001;**289**:1328–33.
21 **Fujita H**, Fujishima H, Morii T, *et al.* Effect of metformin on adipose tissue resistin expression in db/db mice. *Biochem Biophys Res Commun* 2002;**298**:345–9.
22 **Way JM**, Gorgun CZ, Tong Q, *et al.* Adipose tissue resistin expression is severely suppressed in obesity and stimulated by peroxisome proliferator-activated receptor gamma agonists. *J Biol Chem* 2001;**276**:25651–3.
23 **Lee HM**, Wang G, Englander EW, *et al.* Ghrelin, a new gastrointestinal endocrine peptide that stimulates insulin secretion: enteric distribution, ontogeny, influence of endocrine, and dietary manipulations. *Endocrinology* 2002;**143**:185–90.
24 **Lawrence CB**, Snape AC, Baudoin FM, *et al.* Acute central ghrelin and GH secretagogues induce feeding and activate brain appetite centers. *Endocrinology* 2002;**143**:155–62.
25 **Inui A.** Cancer anorexia-cachexia syndrome: Current issues in research and management. *CA-Cancer J Clin* 2002;**52**:72–91.
26 **Fujimura M**, Inui A. Peptidergic regulation of gastrointestinal motility in rodents. *Peptides* 2000;**21**:1565–82.
27 **Dresner A**, Laurent D, Marcucci M, *et al.* Effects of free fatty acids on glucose transport and IRS-1-associated phosphatidylinositol 3-kinase activity. *J Clin Invest* 1999;**103**:253–9.
28 **Jouven X**, Charles MA, Desnos M, *et al.* Circulating nonesterified fatty acid level as a predictive risk factor for sudden death in the population. *Circulation* 2001;**104**:756–61.
29 **Bado A**, Levasseur S, Attoub S, *et al.* The stomach is a source of leptin. *Nature* 1998;**394**:790–2.
30 **Hansen TK**, Dall R, Hosoda H, *et al.* Weight loss increases circulating levels of ghrelin in human obesity. *Clin Endocrinol* 2002;**56**:203–6.
31 **Shuto Y**, Shibasaki T, Otagiri A, *et al.* Hypothalamic growth hormone secretagogue receptor regulates growth hormone secretion, feeding, and adiposity. *J Clin Invest* 2002;**109**:1429–36.

0013-7227/00/$03.00/0
Endocrinology

Vol. 141, No. 11
Printed in U.S.A.

The Novel Hypothalamic Peptide Ghrelin Stimulates Food Intake And Growth Hormone Secretion.

A.M.WREN, C.J.SMALL, H.L.WARD, K.G.MURPHY, C.L.DAKIN, S.TAHERI, A.R.KENNEDY, G.H. ROBERTS, D.G.A.MORGAN, M.A.GHATEI, S.R.BLOOM

Endocrine Unit, Imperial College School of Medicine, Hammersmith Hospital, Du Cane Rd, London W12 ONN, UK.

Ghrelin, a novel 28 amino acid peptide found in hypothalamus and stomach, was recently identified as the endogenous ligand for the growth hormone secretagogue receptor (GHS-R). We have now found that both intracerebroventricular (ICV) and intraperitoneal (i.p.) administration of ghrelin in freely feeding rats stimulated food intake. The onset of increased feeding was rapid and after ICV administration was sustained for 24 hours. Following ICV administration of 3nmol ghrelin, the duration and magnitude of the feeding stimulation was similar to that following 5nmol neuropeptide Y (NPY). Plasma growth hormone (GH) concentration increased following both ICV and i.p. administration of ghrelin. Release of adrenocorticotrophic hormone (ACTH) was stimulated and thyroid stimulating hormone (TSH) inhibited following ICV administration of ghrelin. These data suggest a possible role for the newly identified endogenous hypothalamic peptide, ghrelin, in stimulation of feeding and growth hormone secretion.

Introduction

In 1984 a hexapeptide, developed from an enkephalin analogue programme, was found to be a potent growth hormone secretagogue (1). Subsequently, considerable structure activity work was performed and a number of orally active agents developed. Synthetic growth hormone secretagogues (GHSs) act at the growth hormone secretagogue receptor (GHS-R) to stimulate secretion of growth hormone (GH) and to a lesser extent prolactin and ACTH in several species including humans (1;2). GHSs act both in the hypothalamus (3-5) and directly on the anterior pituitary (1). During the development of synthetic GHSs, weight gain was noted following chronic administration in immature rodents (1). The GHS-R was identified as a G-protein coupled receptor expressed in the pituitary and hypothalamus (6-8). Ghrelin is the recently identified endogenous ligand for this receptor.

In 1999 ghrelin, a novel 28 amino acid peptide, was purified from rat stomach and subsequently cloned in rats and humans (9). Rat and human ghrelin differ by only two amino acid substitutions, suggesting an important physiological role. In common with the synthetic GHSs, ghrelin releases GH following i.v. administration and from primary pituitary cell cultures. Ghrelin immunoreactivity has been detected in the hypothalamic arcuate nucleus (ARC) and in the stomach. GHS-R expression has also been demonstrated in the ARC in NPY and growth hormone releasing hormone (GHRH) neurones (10;11). Furthermore, activation of ARC neurones, shown by induction of c-FOS protein expression, occurs following both systemic and central administration of GHSs (12). Distinct sub-populations of these c-FOS positive cells co-express NPY and GHRH (3;13).
We have therefore examined the effect of intracerebroventricular (ICV) and peripheral (i.p.) administration of ghrelin on food intake and the hypothalamo-pituitary axes.

Methods

Materials
Ghrelin was a gift from Dr Stan Moore at Penninsula Laboratories (St. Helens, Merseyside, UK) and growth hormone releasing peptide 6 (GHRP-6), a synthetic hexapeptide growth hormone secretagogue (1), was purchased from Penninsula Laboratories.
Animals Male Wistar rats (Imperial College School of Medicine, London, UK) weighing 200-300g were maintained in individual cages under controlled temperature (21-23°C) and light (12h light, 12h dark) with *ad libitum* access to food (RM1 diet, SDS UK Ltd.) and water.
Received 07/19/00.

ICV cannulation and injections. For ICV studies, rats were implanted with permanent 22-gauge stainless steel cannulae projecting into the third cerebral ventricle and peptides administered as previously described (14;15). For i.p injections, all compounds were dissolved in 0.9% saline and administered in 0.5ml volume.
Study 1A – Investigation of the effect of i.p. ghrelin on food intake in freely fed rats. Freely fed rats (n=12-20) were injected with saline, ghrelin (3, 10 or 30 nmol) or GHRP-6 (3, 10 or 30 nmol) in the early light phase (09:00-10:00). Immediately after injection rats were returned to cages containing a known amount of chow. Food was reweighed at 1, 2, 4, 8 and 24 hrs post-injection. Behaviour was observed during the first 2 h.
Study 1B – Investigation of the effect of repeated i.p. ghrelin administration on food intake in freely fed rats. Freely fed rats (n = 11-19) received a first i.p. injection of either saline or ghrelin 30 nmol in the early light phase and food intake was measured at 1, 2 and 4 hrs post-injection. Four hours after the first injection a second i.p. injection of either saline or ghrelin 30 nmol was given and food intake measured at 1 and 2 hrs post-injection. The four study groups investigated comprised animals that had received the following injections (saline/saline; saline/ghrelin; ghrelin/saline; ghrelin/ghrelin).
Study 2 – Investigation of the effects of ICV ghrelin and GHRP-6 on food intake in freely fed animals. Freely fed animals were injected with saline, ghrelin (0.3, 1.0, 3.0 or 10 nmol, n=9), GHRP-6 (0.3, 1.0 or 3.0 nmol, n=9), or NPY 5 nmol (n=7). Immediately after injection, animals were returned to cages containing a known amount of chow. Food was reweighed at 1, 2, 4, 8 and 24 hrs post-injection and behaviour observed.
Study 3A – Investigation of the effect of i.p. ghrelin on pituitary hormones. Rats were injected with ghrelin 30nmol or saline (09:00-10:00), chosen as the most effective dose in study 1A and by reference to the published effective molar doses of GHRP-6 (1). Rats were killed by decapitation at 5, 15, 30 and 60 minutes post-injection (n=8) and trunk blood collected into plastic lithium heparin and EDTA tubes containing 0.6mg aprotinin (Bayer, Haywards Heath, UK). Plasma was separated by centrifugation, frozen and stored at –20°C.
Study 3B – Investigation of the effect of repeated i.p. ghrelin administration on pituitary hormones. Rats (n=11-19) received an i.p. injection of either saline or ghrelin 30 nmol at 09:00-10:00 and 4 hrs later received a second i.p. injection of either saline or ghrelin 30 nmol. Animals were decapitated 15 min after the second injection and plasma collected. This time point was investigated as the peak GH response to a single i.p. ghrelin injection occurred at 15 min.
Study 4 – Investigation of the effect of ICV ghrelin on pituitary hormones
Rats were injected with ghrelin 2nmol or saline (n=10) at 09:00 to 10:00 and decapitated 20 min post-injection and trunk blood collected as above. The 20 min time-point was chosen based on the results study 3A and previously published effects of GHRP-6 (16)

Radioimmunoassays

Plasma levels of GH, prolactin, TSH and Luteinizing Hormone (LH) were assayed using reagents and methods provided by the NIDDK and the National Hormone and Pituitary Program (Dr. A. Parlow, Harbor University of CA, Los Angeles Medical Center) as previously described (17). ACTH was measured using a solid-phase immunoradiometric assay (Biogenesis, Poole, Dorset, U.K.).

Statistics

Results are shown as mean values ± SEM. Unpaired Student's t-test was used for comparisons between two treatment groups. Analysis of variance with *post hoc* Least Significant Differences (LSD) test (Systat, Evanston, IL) was used for comparisons between three or more treatment groups. $P < 0.05$ was considered to be statistically significant.

Results

Study 1 - Feeding response to i.p. ghrelin and GHRP-6 in freely fed rats. Peripheral injection (i.p.) of ghrelin or GHRP-6 potently stimulated food intake in a dose dependent manner (figure 1a). This effect was only observed for one hour post-injection. No significant difference was observed between treatment and control groups at any subsequent time-point. Ghrelin-induced feeding was not attenuated following repeated administration (figure 1b). Rats that had received i.p. ghrelin 30 nmol or saline in the early light phase (09:00-10:00) exhibited a second significant feeding response to i.p ghrelin 30 nmol administered 4 hrs later. The magnitude of the second feeding response did not differ significantly between rats pre-treated with saline or ghrelin. As observed in the first study, feeding was only stimulated for one hour after each injection of ghrelin (data not shown). No adverse behaviour was observed following i.p. injections.

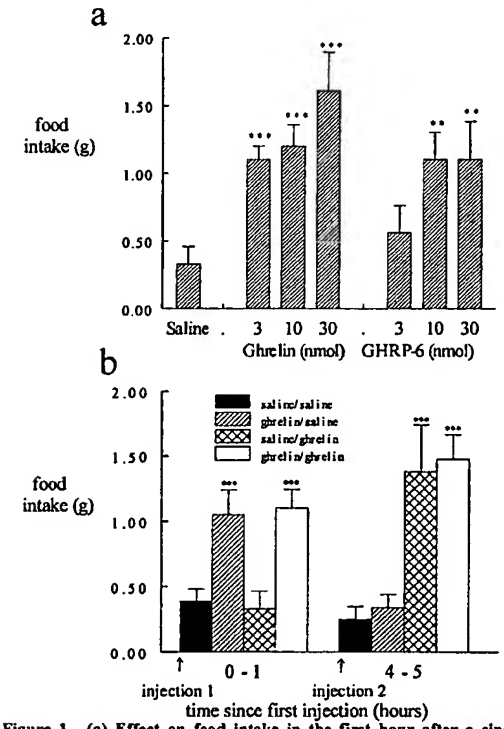


Figure 1. (a) Effect on food intake in the first hour after a single i.p. injection of ghrelin, GHRP-6 or saline in the early light phase (grams per rat ± SEM). (b) Effect on food intake of an i.p. injection of saline or ghrelin 30 nmol in the early light phase followed by a repeated i.p. injection of saline or ghrelin four hours later. Animals received one of the following pairs of injection; saline/saline, saline/ghrelin, ghrelin/saline or ghrelin/ghrelin. Food intake is shown in the first hour after each injection. Significance levels shown for comparison with saline/saline injected animals. ** $p < 0.01$, *** $p \leq 0.001$.

Study 2 - Investigation of the effect of ICV ghrelin and GHRP-6 on feeding in freely fed rats. ICV injection of ghrelin or GHRP-6 stimulated feeding in a dose-dependent manner. This effect was greatest at 1 hr for both compounds. All doses tested produced significant stimulation of feeding (figure 2a) but the effect of the endogenous GHS-R agonist ghrelin was more sustained than that of GHRP-6. Ghrelin increased 24-hr food intake to 140% of saline control, whereas GHRP-6 significantly stimulated food intake up to 8 h, but did not significantly increase 24-h food intake (figure 2b; 24-hr food intake saline 18.5 ± 2.0g; ghrelin 3nmol 25.8 ± 1.1g, p=0.01; GHRP-6 3nmol 21.5 ± 1.8 g, p=0.3). No additional increase in feeding was seen following administration of 10 nmol compared to 3 nmol of ghrelin. The increase in food intake following ICV injection of ghrelin 3nmol was comparable to that observed following ICV injection of NPY 5nmol (figure 2b; 24-h food intake 25.3 ± 1.8, p=0.03; 137% of the saline group). No adverse behaviour was observed.

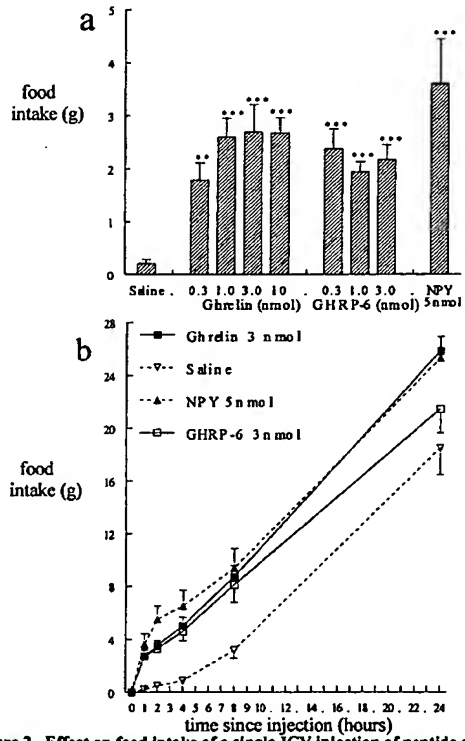


Figure 2. Effect on food intake of a single ICV injection of peptide or saline given in the early light phase. (a) Food intake in the first hour post-injection (grams per rat ± SEM). All treatment groups were significantly different from saline, ** $p < 0.01$, *** $p \leq 0.001$. (b) Cumulative food intake during 24 hours post-injection of peptide or saline. Food intake in all treatment groups (ghrelin 3nmol, NPY 5nmol and GHRP-6 3nmol) was significantly greater than saline at all time points up to 8 hours ($p \leq 0.001$). Food intake was significantly greater than saline at 24 hours in ghrelin 3nmol and NPY 5nmol treated rats ($p < 0.05$), but not in GHRP-6 treated rats ($p = 0.3$).

Study 3 - Investigation of the effect of i.p. ghrelin on plasma pituitary hormones. Plasma GH was potently stimulated at 15 minutes following a single i.p. injection of ghrelin 30 nmol

(figure 3: ghrelin 30nmol 234.3 + 32.5 vs. saline-treated 85.8 + 32.5 ng/ml, p=0.008). No significant difference from the saline control group was seen in plasma LH, TSH or prolactin at any time point (data not shown). In study 3B the effect of repeated i.p. injection of ghrelin 30 nmol on plasma GH was investigated. The plasma GH response was measured 15 minutes after the second i.p. injection. There was no significant difference in the plasma GH of rats that received ghrelin/ghrelin (GH 192.2 + 16.2 ng/ml) compared to rats that received saline/ghrelin (GH 177.8 + 31.2 ng/ml; p=0.6). Both of these treatments produced a significant rise in plasma GH compared to saline/saline treated animals (GH 46.0 + 12.0 ng/ml; $p < 0.001$)

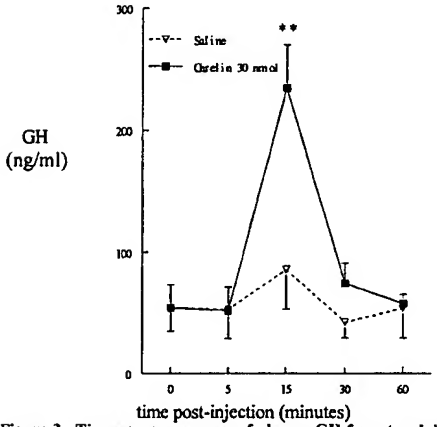


Figure 3. Time-course response of plasma GH from trunk blood collected from rats at 5, 15, 30 and 60 minutes after i.p. injection of either saline or ghrelin 30nmol. ** $p < 0.01$ for comparison with saline control.

Study 4 - Investigation of the effect of ICV ghrelin on plasma pituitary hormones. A single ICV injection of 2nmol ghrelin, chosen from the feeding dose response curve, potently increased plasma GH (ghrelin 156.2 + 29.4 ng/ml vs. saline 11.1 + 3.1 ng ml; $p < 0.001$) and ACTH (ghrelin 38.2 + 3.9 vs. saline 18.2 + 2.0 ng/ml; $p = 0.005$) at 20 min (figure 4a and b). TSH was suppressed 20 min post-injection of 2nmol ghrelin ICV (ghrelin 0.9 + 0.1 vs. saline 1.6 + 0.2 ng/ml; $p<0.01$ figure 4c). No change was seen in plasma LH or prolactin.

Discussion

We have demonstrated that ghrelin, an endogenous GHS-R agonist, administered either into the central nervous system (ICV) or peripherally (i.p.), stimulates food intake. For example, a single 3 nmol dose of ghrelin given ICV to satiated rats increased 24-hour food intake. The time-course and magnitude of this effect was similar to that observed following ICV administration of 5nmol NPY, one of the most potent orexigenic agents known (18; 19).

Single injections of synthetic GHSs have also been previously reported to cause a short lived increase food intake when administered either systemically or ICV (20-22). In contrast the response to ICV ghrelin is prolonged. Chronic ICV administration of the synthetic GHS-R agonist, KP-102, has been reported to cause weight gain and increase 24-hour food intake (23). However, an increase in 24-hour food intake was not observed until the second day of administration. As immature rats were used, KP-102 would be expected to stimulate significant growth and the delayed hyperphagia may be secondary to the increased energy requirements of accelerated linear growth. In our present study, the immediate and sustained increase in food intake caused by ghrelin suggests a direct orexigenic action.

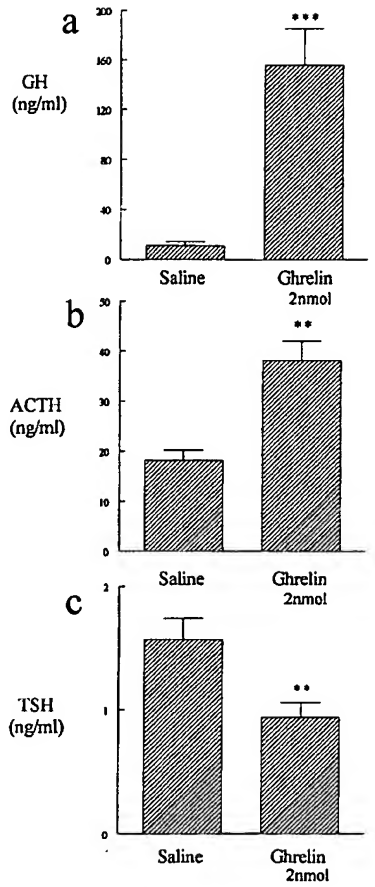


Figure 4. Effect of saline or ghrelin 2nmol ICV on plasma (a) GH, (b) ACTH and (c) TSH at 20 minutes post-injection. Significance levels for comparison with saline, ** $p < 0.01$, *** $p < 0.001$.

It has been previously demonstrated that NPY neurones in the ARC express the GHS-R (10) and express c-FOS following systemic or ICV administration of GHSs (3). Within the ARC, the sub-population of NPY neurones that project to the paraventricular nucleus (PVN) are thought to be important in the control of feeding (18). Retrograde labelling studies suggest that less than 5% of the ARC neurones expressing c-FOS following GHS administration project to the PVN (24). Also approximately 50% of ARC neurones expressing c-FOS following GHS administration do not co-express NPY (3). Thus it is unlikely, but possible, that ghrelin exerts its orexigenic action via activation of NPY neurones in the ARC.

A proportion of GHRH positive neurones express the GHS-R (10) and express c-FOS following GHS administration (3;13). The effects of GHRH on feeding are not well established. Nanomolar doses of GHRH administered ICV have been shown to decrease food intake (25). However, a short-lived increase in food intake has been observed following ICV administration of picomolar doses of GHRH in fasted rats (26). The increase in food intake following acute ICV administration of the synthetic GHS-R agonist KP-102 was unaffected by pre-treatment with a GHRH antagonist, at a dose that completely blocked the feeding response to ICV GHRH (20). Therefore, it is unlikely that ghrelin exerts its orexigenic action via GHRH. The

ability of i.p administration of ghrelin to stimulate feeding is intriguing, as ghrelin is expressed at high levels in the endocrine cells of the stomach (9). Endogenous ghrelin has n-octanoylation of the serine-3 residue which is essential for its GH stimulating action. Hypothalamic arcuate neurones, located close to the blood brain barrier, express GHS-R and are activated following systemic administration of GHSs. We have demonstrated a rapid increase in food intake following systemic administration of ghrelin. It is possible that the fatty acid moiety facilitates passage across the blood brain barrier resulting in hypothalamic actions of systemic ghrelin. Unlike ghrelin, most other hypothalamic peptides, that alter feeding when administered ICV, are ineffective when administered systemically (15;26). Therefore, blockade of endogenous ghrelin may represent a novel target for future treatment of obesity.

We have demonstrated that ghrelin potently stimulated GH release when administered into the CNS or peripherally. The effect of systemic ghrelin on GH was rapid, peaking at 15 minutes, and short lived, returning to baseline by 30 minutes. This is in keeping with reported GH profiles following systemic GHS administration (1). Although GHSs release GH from pituitary cells *in vitro* (1), action at the hypothalamic level is essential for maximal GH response to GHSs *in vivo*. The growth hormone response to systemic administration of GHSs is markedly attenuated by bilateral ARC lesions in rodents (4) and in humans with hypothalamo-pituitary disconnection (5). The potent GH stimulation seen following i.p. ghrelin in our study may be due to action at both pituitary and hypothalamic levels.

The inhibition of TSH by ICV ghrelin may represent an adaptive response to promote feeding and reduce energy expenditure in times of limited nutrition. This effect is also seen with Agouti related protein (27), another hypothalamic orexigenic agent.

In summary we describe the actions of the novel peptide ghrelin on the hypothalamo-pituitary hormonal axes and on food intake. We have demonstrated that both systemic and ICV administration of ghrelin increased plasma GH. The efficacy of ICV ghrelin suggests that an important component of the GH stimulatory effect of ghrelin is at the hypothalamic level. Ghrelin, administered ICV or systemically, potently increases food intake. Further investigation may identify an important role for endogenous ghrelin in the control of food intake and energy balance.

Acknowledgements

We thank the Medical Research Council (MRC) for program grant support (G7811974). A.W., H.W. and S.T. are Wellcome Trust clinical training fellows. C.D. is funded by the British Diabetic Association and K.M. by the M.R.C.

Reference List

1. **Bowers CY, Momany FA, Reynolds GA, Hong A** 1984 On the in vitro and in vivo activity of a new synthetic hexapeptide that acts on the pituitary to specifically release growth hormone. Endocrinology 114:1537-1545

2. **Arvat E, Di Vito L, Maccagno B, Broglio F, Boghen MF, Deghenghi R, Camanni F, Ghigo E** 1997 Effects of GHRP-2 and hexarelin, two synthetic GH-releasing peptides, on GH, prolactin, ACTH and cortisol levels in man. Comparison with the effects of GHRH, TRH and hCRH. Peptides 18:885-891

3. **Dickson SL, Luckman SM** 1997 Induction of c-fos messenger ribonucleic acid in neuropeptide Y and growth hormone (GH)-releasing factor neurons in the rat arcuate nucleus following systemic injection of the GH secretagogue, GH-releasing peptide-6. Endocrinology 138:771-777

4. **Nakagawa T, Ukai K, Ohyama T, Koida M, Okamura H** 1996 Effects of the synthesized growth hormone releasing peptide, KP-102, on growth hormone release in sodium glutamate monohydrate-treated low growth rats. Life Sci 59:705-712

5. **Popovic V, Damjanovic S, Micic D, Djurovic M, Dieguez C, Casanueva FF** 1995 Blocked growth hormone-releasing peptide (GHRP-6)-induced GH secretion and absence of the synergic action of GHRP-6 plus GH-releasing hormone in patients with hypothalamopituitary disconnection: evidence that GHRP- 6 main action is exerted at the hypothalamic level. J Clin Endocrinol Metab 80:942-947

6. **Howard AD, Feighner SD, Cully DF, Arena JP, Liberator PA, Rosenblum CI, Hamelin M, Hreniuk DL, Palyha OC, Anderson J, Paress PS, Diaz C, Chou M, Liu KK, McKee KK, Pong SS, Chaung LY, Elbrecht A, Dashkevicz M, Heavens R, Rigby M, Sirinathsinghji DJS, Dean DC, Melillo DG, van der Ploeg LH** 1996 A receptor in pituitary and hypothalamus that functions in growth hormone release [see comments]. Science 273:974-977

7. **McKee KK, Palyha OC, Feighner SD, Hreniuk DL, Tan CP, Phillips MS, Smith RG, van der Ploeg LH, Howard AD** 1997 Molecular analysis of rat pituitary and hypothalamic growth hormone secretagogue receptors. Mol Endocrinol 11:415-423

8. **Guan XM, Yu H, Palyha OC, McKee KK, Feighner SD, Sirinathsinghji DJ, Smith RG, van der Ploeg LH, Howard AD** 1997 Distribution of mRNA encoding the growth hormone secretagogue receptor in brain and peripheral tissues. Brain Res Mol Brain Res 48:23-29

9. **Kojima M, Hosoda H, Date Y, Nakazato M, Matsuo H, Kangawa K** 1999 Ghrelin is a growth-hormone-releasing acylated peptide from stomach. Nature 402:656-660

10. **Willesen MG, Kristensen P, Romer J** 1999 Co-localization of growth hormone secretagogue receptor and NPY mRNA in the arcuate nucleus of the rat. Neuroendocrinology 70:306-316

11. **Tannenbaum GS, Lapointe M, Beaudet A, Howard AD** 1998 Expression of growth hormone secretagogue-receptors by growth hormone-releasing hormone neurons in the mediobasal hypothalamus. Endocrinology 139:4420-4423

12. **Dickson SL, Leng G, Dyball RE, Smith RG** 1995 Central actions of peptide and non-peptide growth hormone secretagogues in the rat. Neuroendocrinology 61:36-43

13. **Kamegai J, Hasegawa O, Minami S, Sugihara H, Wakabayashi I** 1996 The growth hormone-releasing peptide KP-102 induces c-fos expression in the arcuate nucleus. Brain Res Mol Brain Res 39:153-159

14. **Ong H, McNicoll N, Escher E, Collu R, Deghenghi R, Locatelli V, Ghigo E, Muccioli G, Boghen M, Nilsson M** 1998 Identification of a pituitary growth hormone-releasing peptide (GHRP) receptor subtype by photoaffinity labeling. Endocrinology 139:432-435

15. **Turton MD, O'Shea D, Gunn I, Beak SA, Edwards CM, Meeran K, Choi SJ, Taylor GM, Heath MM, Lambert PD, Wilding JP, Smith DM, Ghatei MA, Herbert J, Bloom SR** 1996 A role for glucagon-like peptide-1 in the central regulation of feeding [see comments]. Nature 379:69-72

16. **Fairhall KM, Mynett A, Robinson IC** 1995 Central effects of growth hormone-releasing hexapeptide (GHRP-6) on growth hormone release are inhibited by central somatostatin action. J Endocrinol 144:555-560

17. **Todd JF, Small CJ, Akinsanya KO, Stanley SA, Smith DM, Bloom SR** 1998 Galanin is a paracrine inhibitor of gonadotroph function in the female rat. Endocrinology 139:4222-4229

18. **Stanley BG, Kyrkouli SE, Lampert S, Leibowitz SF** 1986 Neuropeptide Y chronically injected into the hypothalamus: a powerful neurochemical inducer of hyperphagia and obesity. Peptides 7:1189-1192

19. **O'Shea D, Morgan DG, Meeran K, Edwards CM, Turton MD, Choi SJ, Heath MM, Gunn I, Taylor GM, Howard JK, Bloom CI, Small CJ, Haddo O, Ma JJ, Callinan W, Smith DM, Ghatei MA, Bloom SR.**1997 Neuropeptide Y induced feeding in the rat is mediated by a novel receptor. Endocrinology 138:196-202

20. **Okada K, Ishii S, Minami S, Sugihara H, Shibasaki T, Wakabayashi I** 1996 Intracerebroventricular administration of the growth hormone-releasing peptide KP-102 increases food intake in free-feeding rats. Endocrinology 137:5155-5158

21. **Torsello A, Luoni M, Schweiger F, Grilli R, Guidi M, Bresciani E, Deghenghi R, Muller EE, Locatelli V** 1998 Novel hexarelin analogs stimulate feeding in the rat through a mechanism not involving growth hormone release. Eur J Pharmacol 360:123-129

22. **Locke W, Kirgis HD, Bowers CY, Abdoh AA** 1995 Intracerebroventricular growth-hormone-releasing peptide-6 stimulates eating without affecting plasma growth hormone responses in rats. Life Sci 56:1347-1352

23. **Kuriyama H, Hotta M, Wakabayashi I, Shibasaki T** 2000 A 6-day intracerebroventricular infusion of the growth hormone- releasing peptide KP-102 stimulates food intake in both non-stressed and intermittently-stressed rats. Neurosci Lett 282:109-112

24. **Honda K, Bailey AR, Bull PM, Macdonald LP, Dickson SL, Leng G** 1999 An electrophysiological and morphological investigation of the projections of growth hormone-releasing peptide-6-responsive neurons in the rat arcuate nucleus to the median eminence and to the paraventricular nucleus. Neuroscience 90:875-883

25. **Tanaka Y, Egawa M, Inoue S, Takamura Y** 1991 Effect of hypothalamic administration of growth hormone-releasing factor (GRF) on feeding behavior in rats. Brain Res 558:273-279

26. **Vaccarino FJ, Bloom FE, Rivier J, Vale W, Koob GF** 1985 Stimulation of food intake in rats by centrally administered hypothalamic growth hormone-releasing factor. Nature 314:167-168

27. **Kim MS, Small CJ, Stanley SA, Morgan DG, Seal LJ, Kong WM, Edwards CM, Abusnana S, Sunter D, Ghatei MA, Bloom SR** 2000 The central melanocortin system affects the hypothalamo-pituitary thyroid axis and may mediate the effect of leptin. J Clin Invest 105:1005-1011

Expedited Articles

Structure−Function Studies on the New Growth Hormone-Releasing Peptide, Ghrelin: Minimal Sequence of Ghrelin Necessary for Activation of Growth Hormone Secretagogue Receptor 1a[⊥]

Maria A. Bednarek,*,# Scott D. Feighner,† Sheng-Shung Pong,† Karen Kulju McKee,† Donna L. Hreniuk,† Maria V. Silva,‡ Vivien A. Warren,‖ Andrew D. Howard,† Lex H. Y. Van der Ploeg,† and James V. Heck#

Departments of Medicinal Chemistry, Metabolic Disorders, Drug Metabolism, and Membrane Biochemistry and Biophysics. Merck Research Laboratories, R50G-141, P.O. Box 2000, Rahway, New Jersey 07065

Received April 17, 2000

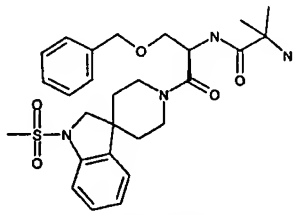
The recently discovered growth hormone secretagogue, ghrelin, is a potent agonist at the human growth hormone secretagogue receptor 1a (hGHSR1a). To elucidate structural features of this peptide necessary for efficient binding to and activation of the receptor, several analogues of ghrelin with various aliphatic or aromatic groups in the side chain of residue 3, and several short peptides derived from ghrelin, were prepared and tested in a binding assay and in an assay measuring intracellular calcium elevation in HEK-293 cells expressing hGHSR1a. Bulky hydrophobic groups in the side chain of residue 3 turned out to be essential for maximum agonist activity. Also, short peptides encompassing the first 4 or 5 residues of ghrelin were found to functionally activate hGHSR1a about as efficiently as the full-length ghrelin. Thus the entire sequence of ghrelin is not necessary for activity: the Gly-Ser-Ser(*n*-octanoyl)-Phe segment appears to constitute the "active core" required for agonist potency at hGHSR1a.

Introduction

The pulsatile release of growth hormone (GH) from the pituitary somatotrophs is regulated by two hypothalamic neuropeptides: growth hormone-releasing hormone (GHRH) and somatostatin (SST). The first peptide stimulates release of GH, whereas SST inhibits secretion of the same hormone.[1,2] Release of GH from the pituitary somatotrophs can also be controlled by the synthetic growth hormone-releasing peptides (GHRPs). A hexapeptide, His-D-Trp-Ala-Trp-D-Phe-Lys-amide (GHRP-6), was the first synthetic peptide which released GH from somatotrophs in a dose-dependent manner in several species including humans.[3] From the subsequent chemical studies on GHRP-6, other potent growth hormone secretagogues (GHSs) resulted: GHRP-1, GHRP-2, and hexarelin, also extensively studied in vitro and in vivo.[4−6]

GHRP-1	Ala-His-D-(2')-Nal-Ala-Trp-D-Phe-Lys-NH_2
GHRP-2	D-Ala-D-(2')-Nal-Ala-Trp-D-Nal-Lys-NH_2
Hexarelin	His-D-2-MeTrp-Ala-Trp-D-Phe-Lys-NH_2

These synthetic GHSs stimulate secretion of GH by a mechanism different from that of GHRH, but like GHRH, they functionally antagonize release of SST from the pituitary and hypothalamus.[3−6] The low oral bioavailability (<1%) of the peptidyl GHSs stimulated a search for nonpeptide compounds mimicking the action of GHRP-6 in the pituitary. Shortly thereafter, several benzolactams and spiroindans were reported to stimulate GH release in various animal species and in humans.[7−9] A small spiroindan with improved oral bioavailability, *N*-[1(*R*)-[(1,2-dihydro-1-methanesulfonylspiro[3*H*-indole-3,4'-piperidin]-1'-yl)carbonyl]-2-(phenylmethoxy)ethyl]-2-amino-2-methylpropanamide methanesulfonate (MK-0677), emerged as one of the most potent synthetic GHSs.[8]



MK-0677

The actions of the above-mentioned GHSs (both peptides and nonpeptides) are mediated by a specific GHS receptor.[10,11] This receptor is present in the pituitary and hypothalamus of various mammalian species (GHSR1a) and it is distinct from the GHRH receptor. The GHS receptor was also detected[9−13] in other areas of the central nervous system (CNS) and in peripheral tissues, for instance adrenal and thyroid glands, heart, lung, kidney, and skeletal muscles. A

⊥ Dedicated to Prof. Dr. Miklos Bodanszky on the occasion of his 85th birthday.

* To whom correspondence should be addressed. Tel: (732) 594-4798. Fax: (732) 594-4773. E-mail: maria_bednarek@merck.com.
# Department of Medicinal Chemistry.
† Department of Metabolic Disorders.
‡ Department of Drug Metabolism.
‖ Department of Membrane Biochemistry and Biophysics.

10.1021/jm0001727 CCC: $19.00
Published on Web 10/26/2000

truncated version of GHSR1a was subsequently reported by scientists from Merck.[10]

The GHS receptors belong to the family of G-protein-coupled receptors. Their activation leads to depolarization and inhibition of potassium channels, to an increase in intracellular concentrations of inositol trisphosphate (IP_3), and to a transient increase in the concentrations of intracellular calcium.[11-13] Thus, it was speculated that the peptidyl and nonpeptidyl GHSs could mimic the actions of yet unidentified endogenous ligand(s) for the GHS receptor(s).

Recently, a 28-amino acid peptide has been isolated from human gut extract.[14] This peptide, ghrelin, was able to induce GH release from primary cultured pituitary cells in a dose-dependent manner without stimulating the release of other pituitary hormones. Injected intravenously into anesthetized rats, ghrelin stimulated pulsatile release of GH.[14] These observations suggested[14] that ghrelin is a specific endogenous ligand for the GHS receptor(s).

Human ghrelin

Ghrelin is also the first peptide isolated from natural sources which possesses the hydroxyl group of one of its serine residues acylated by *n*-octanoic acid. This until now unreported posttranslational modification appears to be necessary for the GH-releasing potency of both human and rat ghrelin.[14] In the GH-releasing assay, the desoctanoyl form of the hormone is at least 100-fold less potent than the parent peptide.[14,15]

The present study aimed at the elucidation of the structural features of human ghrelin which are critical for its interaction with GHSR1a. First, a role of the *n*-octanoyl group in binding to and activation of the cloned hGHSR1a was investigated in detail by evaluation of several analogues of ghrelin in which the hydroxyl group of Ser^3 was acylated with various aliphatic or aromatic acids. Furthermore, the biological importance of the ester group in the side chain of residue 3 was studied through an analogue of ghrelin in which *n*-octanoic acid was coupled to the β-amino group of 2,3-diaminopropionic acid replacing Ser^3. In intact cells, this new analogue of ghrelin, with the *n*-octanoyl group attached to the peptide by an amide bond, should be less susceptible than the parent compound to esterases and acyl migration. Also, analogues esterified at the other three serine residues of ghrelin were tested for their ability to activate hGHSR1a.

Additionally, in an effort to elucidate the smallest segment of human ghrelin which could still activate hGHSR1a, several truncated analogues of this hormone were prepared and evaluated for binding to and activation at hGHSR1a. At this receptor, the agonist potency of the short peptide Gly-Ser-Ser(*n*-octanoyl)-Phe-Leu-NH_2, derived from the N-terminal region of human ghrelin, was equal to that of the full-length ghrelin.

Results

Analogues of human ghrelin (Tables 1-4) were prepared by solid-phase syntheses as described in the Experimental Section. The chromatographically homogeneous peptides **1-26** were analyzed for their chemical integrity by electrospray mass spectrometry, ^1H NMR spectrometry (peptides **21-26**), and chemical sequencing (Edman degradation, peptides **1-22, 24**) (see Experimental Section). In the mass spectra of compounds **1-26**, only molecular ions corresponding to peptides with one *n*-octanoyl group attached were observed. Additionally, the ^1H NMR experiments (COSY and ROESY) on compounds **21-26** showed that *n*-octanoic acid has been coupled to the side chain of Ser^3. This was further supported by the characteristic downfield shift of the signals corresponding to the β-protons of Ser^3, in comparison to the signals of the equivalent protons of Ser^2. Furthermore, chemical sequencing of compounds **1-14**, **18-22**, and **24** yielded negligible amounts of serine in cycle 3 of Edman degradation, thus confirming that Ser^3 in these peptides has been modified. Similarly, upon sequencing of compounds **15-17**, negligible amounts of serine were observed in cycle 2 for compound **15**, in cycle 6 for compound **16**, and in cycle 18 for compound **17**, again due to acylation of the hydroxyl group of the respective serine residues. Taken together, the above observations confirm the presence and exact (expected) location of a single *n*-octanoyl group in the compounds studied.

Peptides **1-26** were evaluated for their binding affinities to the cloned hGHSR1a in a competitive binding assay with [^{35}S]MK-0677 as the radiolabeled ligand and also for their ability to stimulate inositol trisphosphate-coupled mobilization of intracellular calcium in HEK-293 cells expressing hGHSR1a.

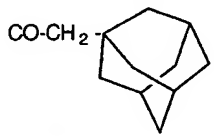
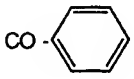
The role of the *n*-octanoyl group in interaction of human ghrelin with hGHSR1a was examined by testing compounds **1-11** (see Table 1) in which the hydroxyl group in the side chain of Ser^3 was acylated by various aliphatic or aromatic acids. Acylation of Ser^3 with hydrophobic acids resembling in size *n*-octanoic acid, such as the unsaturated 2,4,6-octatrienoic acid or the branched 2-propylpentanoic acid or the longer chain 11-undecanoic acid or palmitic acid, yielded compounds **1-4** with agonist potencies similar to that of ghrelin. In contrast, replacement of the *n*-octanoyl group with the substantially smaller acetyl group led to compound **5** which was 20-fold less potent than human ghrelin in the hGHSR1a activation assay. Ghrelin without the *n*-octanoyl group (desoctanoylghrelin) poorly activated hGHSR1a even at micromolar concentrations (compound **6** in Table 1).

To explore possible nonhydrophobic interactions with the receptor, the hydroxyl group of Ser^3 in compounds **7-9** was acylated with aliphatic acids which mimic the extended hydrophobic chain of *n*-octanoic acid, but they also contain bromo, amino, or amido groups. In the hGHSR1a activation assay, analogues with amido and amino groups in the side chain of residue 3 were respectively 5- and 20-fold less potent as agonists at hGHSR1a (compounds **8** and **9** in Table 1), whereas the analogue **7** with a 8-bromooctanoyl group attached to the side chain of Ser^3 retained the potency of the parent compound.

Interestingly, compound **10** with the bulky and rigid hydrophobic 1-adamantaneacetyl group in place of the *n*-octanoyl group in position 3 activated hGHSR1a as

Table 1. Analogues of Human Ghrelin Modified at Position 3

X
|
G S S F L S P E H Q R V Q Q R K E S K K P P A K L Q P R
1 28

No.	X	Binding Assay* IC_{50} (nM)	Functional Assay** EC_{50} (nM)	Functional Assay** % activation at 10 μM relative to ghrelin
human ghrelin	CO-$(CH_2)_6CH_3$	0.25 ± 0.07	32 ± 4.5	100
1	CO-CH=CH-CH=CH-CH=CH-CH_3	0.98 ± 0.36	39 ± 10	108 ± 1
2	CO-$CH(CH_2CH_2CH_3)_2$	0.96 ± 0.05	38 ± 11	103 ± 1
3	CO-$(CH_2)_9CH_3$	0.12 ± 0.03	9.1 ± 6.2	104 ± 3
4	CO-$(CH_2)_{14}CH_3$	0.87 ± 0.17	8.3 ± 0.6	96 ± 11
5	CO-CH_3	> 2000	2000 ± 480	59 ± 13
6	--------	> 10,000	>10,000	41 ± 4
7	CO-$(CH_2)_6CH_2Br$	0.08 ± 0.0	18 ± 0.9	88 ± 7
8	CO-$(CH_2)_2$CO-NH-$(CH_2)_2CH_3$	1020 ± 202	410 ± 120	86 ± 10
9	CO-$(CH_2)_6NH_2$	> 2000	1200 ± 370	68 ± 3
10		0.12 ± 0.05	24 ± 9.5	95 ± 7
11		11 ± 1.5	53 ± 3.2	85 ± 2

*[^{35}S]MK-0677 binding assay. IC_{50} reflects concentration of peptide at 50% specific binding. **Aequorin bioluminescence assay. ED_{50} is the concentration of peptide at 50% maximum calcium accumulation.

efficiently as ghrelin, but compound **11** with a smaller benzoyl group in the same position was 2-fold less potent.

In compounds **12** and **14**, 2,3-diaminopropionic acid was incorporated in place of Ser^3 and the β-amino group of this new residue was acylated with *n*-octanoic acid. Peptides **12** and **14** (Table 2), with the *n*-octanoyl group attached to the side chain of residue 3 through an amide bond, activated hGHSR1a as efficiently as the parent compounds, ghrelin and compound **13** (peptide encompassing 1−14 residues of ghrelin)

Of analogues of ghrelin with the hydroxyl group of Ser^2 or Ser^6 or Ser^{18} selectively acylated by *n*-octanoic acid, compound **15**, with a modified Ser^2, was as potent as ghrelin in the functional assay, whereas compounds **16** and **17**, with a modified Ser^6 and Ser^{18}, respectively, were inactive even at micromolar concentrations (Table 3).

Binding and functional data for truncated analogues of human ghrelin are compiled in Table 4. Shortening of the chain of ghrelin by omission of 5, 10, 14, 18, 23, or 24 residues from its C-terminal end yielded peptides **13** and **18−26** with agonist properties similar to that of the parent compound. The relative binding affinities of the same peptides for the cloned hGHSR1a, however, gradually decreased with the extent of the C-terminal deletions. For example, analogue **13** encompassing residues 1−14 of ghrelin was bound 40-fold weaker than human ghrelin, whereas analogue **21** consisting of only the first 5 residues of ghrelin was 200-fold weaker ligand at the same receptor. The short peptides encompassing residues 1−3, 2−5, 3−5, or 2−4 of ghrelin (compounds **23−26** in Table 4) were poor activators even at micromolar concentrations.

Discussion and Conclusions

Several peptide hormones such as secretin, glucagon, the vasoactive intestinal peptide, and cholecystokinin were detected in and then isolated from the gastrointestinal tract.[16] Of these, cholecystokinin was posttranslationally modified by sulfonylation of the hydroxyl group of Tyr^{27}. It is noteworthy therefore that the recently isolated peptide from gut extracts, ghrelin, is also posttranslationally modified, through acylation of the hydroxyl group of Ser^3 by *n*-octanoic acid. In biological systems, modification of serine residues by

Table 2. Analogues of Human Ghrelin with an Amide Bond in the Side Chain of Residue 3

No.	Compound	Binding Assay* IC_{50} (nM)	Functional Assay** EC_{50} (nM)	Functional Assay** % activation at 10 μM relative to ghrelin
human ghrelin	G S NH-CH(CH$_2$-O-CO(CH$_2$)$_6$CH$_3$)-CO-F L S P E H Q R V Q Q R K E S K K P P A K L Q P R (3)	0.25 ± 0.07	32 ± 4.5	100
12	G S NH-CH(CH$_2$-NH-CO(CH$_2$)$_6$CH$_3$)-CO-F L S P E H Q R V Q Q R K E S K K P P A K L Q P R (3)	0.42 ± 0.12	31 ± 9.8	105 ± 5
13	G S NH-CH(CH$_2$-O-CO(CH$_2$)$_6$CH$_3$)-CO-F L S P E H Q R V Q Q (3)	9.6 ± 1.5	17 ± 4	97 ± 9
14	G S NH-CH(CH$_2$-NH-CO(CH$_2$)$_6$CH$_3$)-CO-F L S P E H Q R V Q Q (3)	8 ± 2.7	38 ± 1.8	102 ± 3

*,**See corresponding footnotes in Table 1.

Table 3. Analogues of Human Ghrelin *n*-Octanoylated at Other Serine Residues

No.	Compound	Binding Assay* IC_{50} (nM)	Functional Assay** EC_{50} (nM)	Functional Assay** % activation at 10 μM relative to ghrelin
human ghrelin	G S S(CO(CH$_2$)$_6$CH$_3$) F L S P E H Q R V Q Q R K E S K K P P A K L Q P R (3)	0.25 ± 0.07	32 ± 4.5	100
15	G S S(CO(CH$_2$)$_6$CH$_3$) F L S P E H Q R V Q Q R K E S K K P P A K L Q P R (2)	48 ± 7.2	42 ± 14	81 ± 14
16	G S S F L S(CO(CH$_2$)$_6$CH$_3$) P E H Q R V Q Q R K E S K K P P A K L Q P R (6)	> 1000	> 10.000	36 ± 1
17	G S S F L S P E H Q R V Q Q R K E S(CO(CH$_2$)$_6$CH$_3$) K K P P A K L Q P R (18)	> 5000	> 10,000	46 ± 2

*,**See corresponding footnotes in Table 1.

acetylation of the hydroxyl group has been occasionally detected,[17] but until now posttranslational acylation of the hydroxyl group with an extended acid such as hydrophobic *n*-octanoic acid has not been reported. This unusual structural feature appears to be necessary for the GH-releasing activity of ghrelin,[14,15] thus suggesting that a hydrophobic interaction between the *n*-octanoyl group and the GHSR1a plays a defining role in molecular recognition. This was supported in this study by agonist potency similar to that of ghrelin of analogues with the unsaturated or branched octanoyl group in position 3 and also of analogues with longer aliphatic chains than the *n*-octanoyl group. For example, peptides with the hydroxyl group of Ser^3 acylated by 11-undecanoic acid or by a 16-carbon chain of palmitic acid (twice as long as *n*-octanoic acid) activated the GHSR1a as efficiently as the parent compound. In contrast, the analogue with the small and thus less hydrophobic acetyl group replacing the *n*-octanoyl group was already a 20-fold less effective agonist than ghrelin, confirming that for efficient activation of hGHSR1a a large hydrophobic group is required in position 3. Kojima and coworkers similarly reported[15] that in the GH-releasing assay, the ghrelin analogue with a *n*-hexanoyl group instead of the *n*-octanoyl group was less potent than ghrelin, whereas in the same assay, the 11-undecanoyl analogue was as potent as the parent compound. The crucial role of hydrophobic interaction in the recognition

Table 4. Truncated Analogues of Human Ghrelin

No.	Compound	Binding Assay* IC_{50} (nM)	Functional Assay** EC_{50} (nM)	Functional Assay** % activation at 10 μM, relative to ghrelin
human ghrelin	$CO(CH_2)_6CH_3$ | G S S F L S P E H Q R V Q Q R K E S K K P P A K L Q P R (1–28)	0.25 ± 0.07	32 ± 4.5	100
18	$CO(CH_2)_6CH_3$ | G S S F L S P E H Q R V Q Q R K E S K K P P A-NH_2 (1–23)	0.16 ± 0.02	15 ± 4.5	100 ± 4
19	$CO(CH_2)_6CH_3$ | G S S F L S P E H Q R V Q Q R K E S-NH_2 (1–18)	0.77 ± 0.18	22 ± 16	92 ± 16
13	$CO(CH_2)_6CH_3$ | G S S F L S P E H Q R V Q Q (1–14)	9.6 ± 1.5	17 ± 4	97 ± 9
20	$CO(CH_2)_6CH_3$ | G S S F L S P E H Q-NH_2 (1–10)	7.1 ± 5.7	20 ± 6	89 ± 17
21	$CO(CH_2)_6CH_3$ | G S S F L-NH_2 (1–5)	55 ± 10	11.5 ± 2.3	96 ± 7
22	$CO(CH_2)_6CH_3$ | G S S F-NH_2 (1–4)	889 ± 72	72 ± 29	91 ± 4
23	$CO(CH_2)_6CH_3$ | Ac-S S F L-NH_2 (2–5)	> 2000	1150 ± 120	30 ± 5
24	$CO(CH_2)_6CH_3$ | G S S-NH_2 (1–3)	> 10,000	>10,000	28 ± 1
25	$CO(CH_2)_6CH_3$ | Ac-S F L-NH_2 (3–5)	> 10,000	2500 ± 1200	29 ± 7
26	$CO(CH_2)_6CH_3$ | Ac-S S F-NH_2 (2–4)	> 10,000	>10,000	28 ± 1

*,**See corresponding footnotes in Table 1.

of ghrelin peptides by GHSR1a was reemphasized by the activity of our analogue of ghrelin in which the *n*-octanoyl group was replaced by a 1-adamantaneacetyl group. This peptide with this rigid hydrophobic substituent at position 3 elicited a response similar to that of analogous peptides with flexible extended hydrocarbon groups. Acylation of the hydroxyl group of Ser^3 with a smaller benzoyl group yielded an analogue about one-half as potent as ghrelin.

Interaction between ghrelin and hGHSR1a seems to be weaker when Ser^3 is modified by acids which mimic the extended hydrophobic chain of *n*-octanoic acid but also possess an amino or amide group which allows formation of ionic or hydrogen bonds, respectively, with the receptor. At least 10-fold lower agonist potency of compounds **8** and **9** at hGHSR1a strongly indicated that polar interaction between the side chain of residue 3 and the receptor is unfavorable for ligand binding and recognition. The corresponding 8-bromooctanoyl analogue was as active as ghrelin.

To evaluate a role of the ester bond in the side chain of residue 3, an analogue isosteric with ghrelin was prepared. The *n*-octanoyl group was attached to the side chain of residue 3 through an amide bond. This experiment was prompted by the expectation that an amide bond in the side chain of residue 3 should be less susceptible to enzyme-catalyzed cleavage than an ester bond. Thus, 2,3-diaminopropionic acid was incorporated in position 3 of the peptide and its β-amino group acylated with *n*-octanoic acid. The agonist potency of this "isosteric ghrelin" was found to be equal to that of ghrelin itself. This indicates that neither detachment nor migration of the *n*-octanoyl group play a role in the mechanism of activation of hGHSR1a.

Of the four serine residues present in the sequence of human ghrelin, only one in position 3 is acylated by *n*-octanoic acid, whereas the hydroxyl groups of the neighboring Ser^2 and the distant Ser^6 and Ser^{18} are free. This suggested that residues close to Ser^3 probably direct the esterification. Yet, residues next to Ser^3 might

also be involved in formation of a ligand–receptor complex, either by directly interacting with the receptor or by contributing to the formation of a biologically active geometry of the N-terminal segment of ghrelin. To test a role of the C-terminal portion of ghrelin in binding and receptor activation, the hydroxyl group of Ser^6 or Ser^{18} was selectively acylated with *n*-octanoic acid. Analogues with the *n*-octanoyl group attached to the side chain of Ser^6 or Ser^{18} had poor activity, even at micromolar concentrations. This suggested that mainly the N-terminal part of ghrelin is involved in molecular recognition. The *n*-octanoyl Ser^2 analogue, however, was equipotent to ghrelin, revealing that the GHS receptor does not recognize the exact location of the *n*-octanoyl group in the N-terminal segment of ghrelin; it does not distinguish between Ser(*n*-octanoyl) in position 2 or 3. The same receptor did not recognize peptides unrelated to ghrelin, even when they contained the Ser(*n*-octanoyl) residue. For example, compounds Tyr-Ser(*n*-octanoyl)-Tyr-Arg-NH_2 and Pro-Lys-Phe-Glu-Ala-Val-Glu-Lys-Pro-Gln-Ser(*n*-octanoyl)-NH_2 did not activate hGHSR1a even at micromolar peptide concentrations.

To determine the smallest segment of ghrelin that can elicit a biological response at hGHSR1a similar to that of the parent compound, truncated analogues of ghrelin were evaluated for binding to and activation of hGHSR1a. The N-terminal part of ghrelin with Ser(*n*-octanoyl) in position 3 was preserved, but the chain was gradually shortened by the omission of blocks of several amino acids from the C-terminal end of ghrelin. Interestingly, a peptide encompassing only the first 5 residues of ghrelin, Gly-Ser-Ser(*n*-octanoyl)-Phe-Leu-NH_2, activated the hGHSR1a as efficiently as the full-length ghrelin. The still shorter tetrapeptide, Gly-Ser-Ser(*n*-octanoyl)-Phe-NH_2, was somewhat less potent. Further omission of amino acids from the C-terminus or N-terminus of the N-terminal tetrapeptide yielded compounds inactive at hGHSR1a, even at micromolar concentrations. Thus, the short segment of ghrelin, Gly-Ser-Ser(*n*-octanoyl)-Phe, appears to constitute the essential part of the chain required for activity at hGHSR1a. Although the N-terminal pentapeptide activated hGHSR1a as efficiently as ghrelin, in competition with [^{35}S]MK-0677 for binding to the cloned hGHSR1a, its affinity was about 200-fold lower than that of the full-length compound. This seems to imply that ghrelin(1–5) and MK-0677 bind differently to the cloned hGHSR1a.

Our studies thus confirmed that the *n*-octanoyl group of ghrelin is one of the principal structural features determining its potency at hGHSR1a. For maximum activity bulky, flexible, or rigid hydrophobic groups are needed in the side chain of residue 3, whereas the hydrophilic groups in the same position significantly reduce agonist activity. The ester group, however, is not essential for binding and activity: it can be replaced by an amide group. Our study also showed that the entire sequence of ghrelin is not necessary for activation of hGHSR1a. The short peptides encompassing the first 4 or 5 residues of ghrelin were found to activate the hGHSR1a about as efficiently as the full-length ghrelin, thus implying that the N-terminal Gly-Ser-Ser(*n*-octanoyl)-Phe segment constitutes the "essential core" required for efficient binding to and activation of hGHSR1a.

In summary, we report here the synthesis of short peptides which are potent agonists at the hGHSR1a. The ghrelin-derived peptides investigated in this study should be useful in further studies on the physiological role of ghrelin and also in the design of new GHSs.

Experimental Section

Materials. Fmoc-protected amino acids were obtained from AnaSpec (San Jose, CA), 4-(2′,4′-dimethoxyphenyl-Fmoc-aminomethyl)phenoxy resin from PE Biosystems (Foster City, CA), Boc-7-aminoheptanoic acid from Bachem (King of Prussia, PA) and *n*-octanoic acid, 2,4,6-octatrienoic acid, 2-propylpentanoic acid, 11-undecanoic acid, palmitic acid, 8-bromooctanoic acid, 1-adamanteneacetic acid and benzoic acid from Aldrich (Milwaukee, WI).

Peptide Synthesis, Purification, and Characterization. Elongation of peptidyl chains on 4-(2′,4′-dimethoxyphenyl-Fmoc-aminomethyl)phenoxy resin was performed on a 431A ABI peptide synthesizer. Manufacturer-supplied protocols were applied for coupling of the hydroxybenzotriazole esters of amino acids in *N*-methylpyrrolidone (NMP). The fluorenylmethyloxycarbonyl (Fmoc) group was used as a semipermanent α-amino protecting group, whereas the side chain protecting groups were: *tert*-butyl for serine, trityl for serine in position 3 and for histidine and glutamine, *tert*-butyl ester for glutamic acid, 2,2,4,6,7-pentamethyldihydrobenzofuran-5-sulfonyl (Pbf) for arginine, *tert*-butyloxycarbonyl (Boc) for lysine and for α-amino group of glycine in position 1. The peptidyl resin was then transferred into a vessel and the trityl group from the side chain of Ser^3 was manually removed with 1% trifluoroacetic acid (TFA) in dichloromethane (45 min at room temperature). The peptidyl resin was thoroughly washed and then agitated for 4 h with the 6-fold excess of each 1-ethyl-3-(3-(dimethylamino)propyl)carbodiimide (EDC) and a selected acid in dichloromethane (DCM) or NMP, in the presence of a catalytic amount of 4-(dimethylamino)pyridine (ca. 10 mg). The peptidyl resin was again washed with DCM, NMP, and methanol, dried, and treated with TFA in the presence of scavengers (ca. 3% total of the mixture of water–anisole–triethylsilane, 1:1:1, v/v/v). After 1.5 h, the resin was filtered off, TFA was removed in vacuo and the residue was triturated with ether. The precipitate which formed was filtered off, washed thoroughly with ether, and dried. The crude peptide was analyzed by analytical reverse-phase high-pressure liquid chromatography (RP HPLC) on a C18 Vydac column attached to a Waters 600E system with automatic Wisp 712 injector and 991 photodiode array detector. A standard gradient system of 0–100% buffer B in 30 min (G1) or a gradient of 20–80% buffer B in 30 min (G2) was used for analysis; buffer A was 0.1% trifluoroacetic acid in water and buffer B was 0.1% trifluoroacetic acid in acetonitrile. HPLC profiles were recorded at 210 and 230 nm. Preparative separations were performed on a Waters Delta Prep 4000 system with a semipreparative C18 RP Vydac column. The above-described solvent system of water and acetonitrile, in a gradient of 20–80% buffer B in 60 min (G3) or in a gradient of 0–60% buffer B, was used for separations. The chromatographically homogeneous compounds (purity > 94%) were analyzed by electrospray mass spectrometry (Hewlett-Packard series 1100 MSD spectrometer) and by peptide sequencing (ABI 494 cLC protein sequencer).

The ^{1}H NMR spectra (400 MHz) of compounds **21–26** were recorded on Varian Unity 400 (Varian Inc., California) in CD_3OH (0.13 mL) at 25 °C in 3-mm NMR tubes using a 3-mm indirect detection gradient probe (Nalorac Corp., California). The data processing was performed on the spectrometer. Chemical shifts were reported on the δ scale (ppm) by assigning the residual solvent peak at 3.30 ppm to ^{1}H of methanol. The COSY spectra were acquired with a spectral width of 3597.1 Hz into 1K data points in f_2, with 357

increments in the f_1 dimension, and the 90° pulse was 7.75 μs. The ROESY spectra were acquired with a spectral width of 3597.1 Hz into 1K data points in f_2 and with 358 increments in the f_1 dimension. The delay between the successive pulses was 2 s, the mixing time used was 0.3 s, and the 90° pulse was 7.75 μs.

Filter Binding Assay. Binding of [^{35}S]MK-0677 to crude membranes prepared from HEK 293-aequorin stable cell lines was performed as described in detail in refs 9 and 10. For a 96-well filter binding assay, 0.05 nM [^{35}S]MK-0677 (specific activity ~ 1200 Ci/mmol) was bound to 4 μg of membrane protein/well with or without competing test ligand. The bound membranes were filtered on 0.5% polyethylenimine prewet filters (UniFilter 96 GF/C, Packard #6005174, Meriden, CT). Filters were washed 8 times, dried, and counted with Microscint 20 (Packard #6013621, Meriden, CT). IC_{50} values were determined from three separate assays performed in triplicate.

Aequorin Bioluminescence Functional Assay. The aequorin bioluminescence assay is a reliable test for identifying G-protein-coupled receptors which couple through the Gα protein subunit family consisting of G_q and G_{11} which leads to the activation of phospholipase C, mobilization of intracellular calcium, and activation of protein kinase C. A stable cell line expressing the hGHSR1a and the aequorin reporter protein were used.[18] The assay was performed using a Luminoskan RT luminometer (Labsystems Inc., Gaithersburg, MD) controlled by custom software written for a Macintosh PowerPC 6100. 293AEQ17/GHSR1a cells were cultured for 72 h and the apo-aequorin in the cells was charged for 1 h with coelenterazine (10 μM) under reducing conditions (300 μM reduced glutathione) in ECB buffer (140 mM NaCl, 20 mM KCl, 20 mM HEPES–NaOH, pH 7.4, 5 mM glucose, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 0.1 mg/mL bovine serum albumin). The cells were harvested, washed once in ECB medium, and resuspended to 500 000 cells/mL. 100 mL of cell suspension (corresponding to 5×10^4 cells) was then injected into the test plate containing the ghrelin peptides, and the integrated light emission was recorded over 30 s, in 0.5-s units. 20 μL of lysis buffer (0.1% final Triton X-100 concentration) was then injected and the integrated light emission recorded over 10 s, in 0.5-s units. The "fractional response" values for each well were calculated by taking the ratio of the integrated response to the initial challenge to the total integrated luminescence including the Triton X-100 lysis response. The functional EC_{50} values were measured in three separate assays.

Acknowledgment. The authors thank Dr. McHardy M. Smith (Merck Research Laboratories, Rahway, NJ) and Ms. Theresa L. Wood (Merck Research Laboratories, West Point, PA) for chemical sequencing of ghrelin analogues.

Supporting Information Available: Table S1 with RP HPLC, MS, and peptide sequencing data for compounds **1**–**26**; Tables S2 and S3 with ^{1}H NMR chemical shift assignments for compounds **21**–**26**. This material is available free of charge via the Internet at http://pubs.acs.org.

References

(1) Frohman, L. A.; Jansson, J. O. Growth hormone-releasing factor. *Endocronol. Rev.* **1986**, *7*, 223–253.
(2) Strobl, J. S.; Thomas, M. J. Human growth hormone. *Pharmacol. Rev.* **1994**, *46*, 1–34.
(3) Bowers, C. Y.; Momany, F. A.; Reynolds, G. A.; Hong, A. On the *in vitro* and in vivo activity of a new synthetic hexapeptide that acts on the pituitary to specifically release growth hormone. *Endocrinology* **1984**, *114*, 1537–1545.
(4) Cheng, K.; Chan, W. W.; Barreto, A., Jr.; Convey, E. M.; Smith, R. G. The synergistic effects of His-D-Trp-Ala-Trp-D-Phe-Lys-NH_2 on growth hormone (GH)-releasing factor-stimulated GH release and intracellular adenosine 3′,5′-monophosphate accumulation in rat primary pituitary cell culture. *Endocrinology* **1989**, *124*, 2791–2798.
(5) Bowers, C. Y. Novel GH-releasing peptides. In *Molecular and Clinical Advances in Pituitary Disorders*; Melmed, S., Ed.; Endocrine Research and Education, Inc.: Los Angeles, CA, 1993; pp 153–157.
(6) Deghenghi, R.; Cananzi, M.; Torsello, A.; Battiati, C.; Muller, E. E.; Locatelli, V. GH-releasing activity of hexarelin a new growth hormone releasing peptide in infant and adult rats. *Life Sci.* **1994**, *54*, 1321–1328.
(7) Smith, R. G.; Cheng, K.; Schoen, W. W.; Pong, S.-S.; Hickey, G.; Jacks, T.; Butler, B.; Chan, W. W.-S.; Chaung, L.-Y. P.; Judith, F.; Taylor, J.; Wyvratt, M. J.; Fisher, M. H. A nonpeptidyl growth hormone secretagogue. *Science* **1993**, *260*, 1640–1643.
(8) Patchett, A. A.; Nargund, R. P.; Tata, J. R.; Chen, M.-H.; Barakat, K. J.; Johnston, D. B. R.; Cheng, K.; Chan, W. W.-S.; Butler, B.; Hickey, G.; Jacks, T.; Schleim, K.; Pong, S.-S.; Chaung, L.-Y. P.; Chen, H. Y.; Frazier, E.; Leung, K. H.; Chiu, S.-H. L.; Smith R. G. The design and biological activities of L-163,191 (MK-0677): A potent, orally active growth hormone secretagogue. *Proc. Natl. Acad. Sci. U.S.A.* **1995**, *92*, 7001–7005.
(9) Chen, M.-H.; Steiner, M. G.; Patchett, A. A.; Cheng, K.; Wei, L.; Chan, Q. W.-S.; Butler, B.; Jacks, T. M.; Smith, R. G. Analogues of the orally active growth hormone secretagogue L-162,752. *Bioorg. Med. Chem. Lett.* **1996**, *6*, 2163–2169.
(10) Howard, A. D.; Feighner, S. D.; Cully, D. F.; Arena, J. P.; Liberator, P. L.; Rosenblum, C. I.; Hamelin, M.; Hreniuk, D. L.; Palyha, O. C.; Anderson, J.; Paress, P. S.; Diaz, C.; Chou, M.; Liu, K. K.; McKee, K.-K.; Pong, S.-S.; Chaung, L.-Y.; Elbrecht, A.; Dashkevicz, M.; Heavens, R.; Rigby, M.; Sirinathsinghji, D.; Dean, D. C.; Melillo, D. G.; Patchett, A. A.; Nargund, R.; Griffin, P. R.; DeMartino, J. A.; Gupta, S. K.; Schaeffer, J. A.; Smith, R. G.; Van der Ploeg, L. H. T. A receptor in pituitary and hypothalamus that functions in growth hormone release. *Science* **1996**, *273*, 974–977.
(11) Pong, S.-S.; Chaung, L.-Y. P.; Dean, D. C.; Nargund, R. P.; Patchett, A. A.; Smith, R. G. Identification of a new G protein-linked receptor for growth hormone secretagogues. *Mol. Endocrinol.* **1996**, *10*, 57–61.
(12) Guan, X.-M.; Yu, H.; Palyha O. C.; McKee, K. K.; Feighner, S. D.; Sirinathsinghji, D. J. S.; Smith, R. G.; Van der Ploeg, L. H. T.; Howard, A. D. Distribution of mRNA encoding the growth hormone secretagogue receptor in brain and peripherial tissues. *Mol. Brain Res.* **1997**, *48*, 23–29.
(13) McKee, K. K.; Tan, C. P.; Palyha, O. C.; Liu, J.; Feighner, S. D.; Hreniuk, D. L.; Smith, R. G.; Howard, A. D.; Van der Ploeg, L. H. T. Cloning and characterization of two human G protein-coupled receptor genes (GPR38 and GPR39) related to the growth hormone secretagogue and neurotensin receptors. *Genomics* **1997**, *46*, 426–434.
(14) Kojjima, M.; Hosada, H.; Date, Y.; Nakazato, M.; Matsuo, H.; Kangawa, K. Ghrelin is a growth-hormone-releasing acylated peptide from stomach. *Nature* **1999**, *402*, 656–660.
(15) Kojima, M. Ghrelin is a novel growth hormone releasing acylated peptide from stomach (Abstract). Third International Symposium on Growth Hormone Secretagogues, Keystone, CO, Feb 17–19, 2000.
(16) Mutt, V. Chemistry, Isolation and purification of gastrointestinal peptides. *Biochem. Soc. Trans.* **1980**, *18*, 11–14 and references within.
(17) Olsen, R. K. The quinoxaline depsipeptide antibiotics. In *Chemistry and Biochemistry of Amino Acids, Peptides and Proteins*; Weinstein, B., Ed.; Marcel Dekker, Inc.: New York and Basel, 1983; Vol. 7, pp 1–33.
(18) Button, D.; Brownstein, M. Aequorin-expressing mammalian cell lines used to report Ca^{2+} mobilization. *Cell Calcium* **1993**, *14*, 663–671.

JM0001727

Gut 2005;**54**:18-24

STOMACH

Stomach regulates energy balance via acylated ghrelin and desacyl ghrelin

A Asakawa[1], A Inui[1], M Fujimiya[2], R Sakamaki[3], N Shinfuku[3], Y Ueta[4], M M Meguid[5] and M Kasuga[1]

[1] Division of Diabetes, Digestive and Kidney Diseases, Department of Clinical Molecular Medicine, Kobe University Graduate School of Medicine, Kobe, Japan
[2] Department of Anatomy, Shiga University of Medical Science, Shiga, Japan
[3] International Center for Medical Research, Kobe University Graduate School of Medicine, Kobe, Japan
[4] Department of Physiology, School of Medicine, University of Occupational and Environmental Health, Kitakyushu, Japan
[5] Department of Surgery, SUNY Upstate Medical University, Syracuse, NY, USA

Correspondence to:
Professor A Inui
Division of Diabetes, Digestive and kidney Diseases, Department of Clinical Molecular Medicine, Kobe University Graduate School of Medicine, 7-5-1 Kusunoki-cho, Chuo-ku, Kobe 650–0017, Japan; inui@med.kobe-u.ac.jp

Accepted for publication 14 June 2004

ABSTRACT

TOP
ABSTRACT
METHODS
RESULTS
DISCUSSION
REFERENCES

Background/Aims: The gastric peptide ghrelin, an endogenous ligand for growth-hormone secretagogue receptor, has two major molecular forms: acylated ghrelin and desacyl ghrelin. Acylated ghrelin induces a positive energy balance, while desacyl ghrelin has been reported to be devoid of any endocrine activities. The authors examined the effects of desacyl ghrelin on energy balance.

Methods: The authors measured food intake, gastric emptying, c-Fos expression in the hypothalamus, and gene expression of hypothalamic neuropeptides in mice after administration of desacyl ghrelin. To

explore the effects of long term overexpression of desacyl ghrelin, transgenic mice that overexpressed desacyl ghrelin were created.

Results: Administration of desacyl ghrelin decreased food intake and gastric emptying rate through an action on the paraventricular nucleus and the arcuate nucleus in the hypothalamus. Gene expression of anorexigenic cocaine and amphetamine regulated transcript and urocortin in the hypothalamus was increased by desacyl ghrelin. Desacyl ghrelin overexpressing mice exhibited a decrease in body weight, food intake, and fat pad mass weight accompanied by moderately decreased linear growth. Gastric emptying was also decreased in desacyl ghrelin overexpressing mice.

Conclusions: These findings indicate that in contrast to acylated ghrelin, desacyl ghrelin induces a negative energy balance by decreasing food intake and delaying gastric emptying. The effect is mediated via the hypothalamus. Although derived from the same precursor, the inverse effects of these two peptides suggest that the stomach might be involved as an endocrine organ in the regulation of the energy balance.

Abbreviations: ACSF, artificial cerebrospinal fluid; AGRP, agouti related protein; ARC, arcuate nucleus; CART, cocaine and amphetamine regulated transcript; CRF, corticotrophin releasing factor; FFA, free fatty acids; GHS-R, growth hormone secretagogue receptor; G3PDH, glyceraldehyde 3-phosphate dehydrogenase; ICV, intra-third cerebroventricular(ly); MCH, melanin concentrating hormone; NTS, nucleus tractus solitarius; NMU, neuromedin U; PBS, phosphate buffered saline; POMC, proopiomelanocortin; PVN, paraventricular nucleus; RT-PCR, reverse transcription polymerase chain reaction

Keywords: gastric peptide; hypothalamus; food intake; body weight; transgenic mice

Ghrelin, a 28-amino acid peptide with structural resemblance to motilin, was recently identified in the stomach as an endogenous ligand for growth hormone secretagogue receptor (GHS-R).[1] The ghrelin gene is predominantly expressed in the stomach and ghrelin is secreted into the circulatory system. Two major molecular forms of ghrelin are found in the stomach and plasma: acylated ghrelin, which has n-octanoylated serine in position 3; and desacyl ghrelin.[2] Other minor forms of ghrelin are also present in the stomach and plasma.[3] The n-octanoyl modification of ghrelin appears to be essential for GH releasing activity as endogenous ligand for GHS-R.[1] Acylated ghrelin is involved in the regulation of GH secretion, energy balance, gastrointestinal motility, cardiac performance, and anxiety.[4–8] Expression and secretion of ghrelin are increased by fasting and are reduced by feeding.[4,5] In addition, diet induced weight loss increases plasma ghrelin level in humans.[9] Administered acylated ghrelin induces body weight gain and adiposity by promoting food intake and decreasing fat use or energy expenditure.[4,5,10] On the other hand, desacyl ghrelin has been reported to be devoid of any endocrine activities. However, plasma desacyl ghrelin concentration accounts for more than 90% of total circulating ghrelin.[2,11] We investigated the effects of desacyl ghrelin on energy balance.

METHODS

▲ TOP
▲ ABSTRACT
▪ METHODS
▼ RESULTS
▼ DISCUSSION
▼ REFERENCES

Animal experiments

We used male mice of the ddy strain (34–37 g, 8–9 weeks old; Japan Slc, Shizuoka, Japan). The mice were individually housed in a regulated environment (22 (SEM 2)°C, 55 (SEM 10) % humidity, 12:12 hours light:dark cycle with light on at 7:00am). Food and water were available ad libitum except as otherwise indicated. The mice were used only once each in the experiment. All experiments were approved by our university animal care committee. Mouse desacyl ghrelin and mouse acylated ghrelin were purchased from Phoenix Pharmaceuticals, Inc (Belmont, CA, USA) and the Peptide Institute (Osaka, Japan), respectively. Just before administration, each drug was diluted in 4 μl of artificial cerebrospinal fluid (ACSF) for intra-third cerebroventricular (ICV) injection or in 100 μl physiological saline for intraperitoneal (IP) injection.

ICV substance application

For ICV injection, the mice were anaesthetised with sodium pentobarbital (80–85 mg/kg IP) and placed in a stereotaxic instrument seven days before the experiments. A hole was made in each skull by using a needle inserted 0.9 mm lateral to the central suture and 0.9 mm posterior to the bregma. A 24 gauge cannula bevelled at one end over a distance of 3 mm was implanted into the third cerebral ventricle for ICV injection. The cannula was fixed to the skull with dental cement and capped with silicon without an obtruder. A 27 gauge injection insert was attached to a microsyringe by PE-20 tubing.

Feeding tests

Experiments were started at 10:00am. Before feeding tests, mice were deprived of food for 16 hours with free access to water, or were given free access to food and water. A standard diet (CLEA Japan Inc, Tokyo, Japan) was used. Food intake was measured by subtracting uneaten food from initially premeasured food at 20 minutes, one hour, and two hours after administration and checking for food spillage. Acylated ghrelin was administered intracerebroventricularly (1 nmol/mouse). Desacyl ghrelin was administered intracerebroventricularly (0.1–1 nmol/mouse) or intraperitoneally (0.3–3 nmol/mouse).

Gastric emptying

Before the experiments in gastric emptying, mice were food deprived for 16 hours with free access to water. The fasted mice had free access to preweighed pellets for one hour; they (except desacyl ghrelin-overexpressing mice) were then administered intracerebroventricularly (0.1–1 nmol/mouse) or intraperitoneally (0.3–3 nmol/mouse) with desacyl ghrelin. The mice were deprived of food again for two hours after administration. Food intake was measured by weighing uneaten pellets. Mice were killed by cervical dislocation three hours after the start of experiments. Immediately after, the stomach was exposed by laparotomy, quickly ligated at both the pylorus and cardia, then removed; then the dry content was weighed. Contents were dried by a vacuum freeze drying system (Model 77400, Labconco Corp, Kansas City, MO, USA). Gastric emptying was calculated according to the following formula: gastric emptying (%) = {1 - (dry weight of food recovered from the stomach/weight of food intake)} x100.[12–14]

c-Fos immunohistochemistry

On the day of the study, mice placed on ad libitum access to food and water were administered intraperitoneally with desacyl ghrelin (3 nmol/mouse), acylated ghrelin (3 nmol/mouse), or physiological saline. Food was removed immediately from the cages to prevent induction of c-Fos immunoreactivity resulting from feeding, Ninety minutes after administration, mice were anaesthetised with sodium pentobarbital (80–85 mg/kg IP) and perfused with isotonic phosphate buffered saline (PBS) followed by 4% paraformaldehyde, 0.5% glutaraldehyde, and 0.2% picric acid in 0.1 M phosphate buffer. Brains were removed and postfixed with 4% paraformaldehyde and 0.2% picric acid in 0.1 M phosphate buffer. Brains were cut into 50 μm thick coronal sections in a cryostat. Slices were incubated with rabbit polyclonal anti-c-Fos antibody (Oncogene Science, Cambridge, MA, USA) diluted 1:5000. Slices were then processed for biotinylated goat anti-rabbit IgG and avidin biotin peroxidase complex. Immunoreaction was performed with 0.01% 3,3'-diaminobenzidine, 1% ammonium nickel sulphate, and 0.0003% H_2O_2 solution. Presence of c-Fos immunoreactivity was revealed as a purple precipitate located in the neuronal nuclei. The exact anatomical localisation of c-Fos positive neurons in the brain nuclei was examined using cresyl violet staining, which was performed on the adjacent sections cut through each brain nucleus. The number of cells that showed c-Fos immunoreactivity was counted in paraventricular and arcuate nuclei.

Real-time RT-PCR

Mice were fasted for 12 hours. During the period of fasting, mice, except transgenic mice, were treated with desacyl ghrelin (3 nmol/mouse) or physiological saline every six hours for 12 hours, with the third and final administration at 30 minutes before the mice were killed by cervical dislocation. Immediately after, the hypothalamic block was removed, frozen on dry ice, and stored at –80°C until preparation of real-time reverse transcription polymerase chain reaction (real-time RT-PCR). Using the RNeasy Mini Kit (Qiagen KK, Tokyo, Japan) RNA was isolated from the hypothalamic block. Quantification of mRNA levels was performed with SYBR-green chemistry (Qiagen KK) using a one step RT-PCR reaction on an ABI PRISM 7700 Sequence Detection System purchased from Applied Biosystems Japan, Ltd. (Tokyo, Japan). The reaction was performed under standard conditions recommended by the manufacturer. We used the mouse glyceraldehyde 3-phosphate dehydrogenase (G3PDH) gene as an internal control. All expression data were normalised to G3PDH expression level from the same individual sample. The primers are shown in table 1.

View this table: [in this window] [in a new window]

Table 1 Accession numbers and primers. G3PDH, glyceraldehyde 3-phosphate dehydrogenase

Plasmid construction and production of transgenic mice

Mouse ghrelin cDNA was obtained by RT-PCR from RNA isolated from mouse stomach. The sequence of the ghrelin cDNA was checked by ABI PRISM 3100 Genetic Analyzer purchased from Applied Biosystems Japan, Ltd (Tokyo, Japan). Plasmid pCAGGS ghrelin was constructed by inserting a mouse ghrelin cDNA into the unique *Eco*RI site between the CAG promoter and 3' flanking sequence of the

rabbit ß-globin gene of the pCAGGS expression vector.[15] The pCAGGS expression vector was kindly donated by Professor Junichi Miyazaki (Osaka University, Osaka, Japan). The DNA fragment was excised from its plasmid by digestion with *Sal*I and *Bam*HI, then purified and microinjected into pronuclei of fertilised eggs obtained from BDF1 female mice as reported previously. Transgenic (Tg) mice were usually identified by PCR and Southern blot analyses. Transgenic founders were mated with wild type C57/BL6 mice. The resultant Tg and non-Tg male littermates were used. Eight independent lines of Tg mice were identified with three to 12 copies of the ghrelin transgene. The mouse line (3–3) with the greatest ghrelin expression was used for all studies described in this paper. Selected studies were also performed with another line (11–1) containing 12 copies of the transgene to confirm observations made in the first line. All experiments were approved by the Kobe University Animal Care Committee.

Southern blot analysis

Mouse genomic DNA was isolated from mice tails.[16] Mouse DNA was digested by EcoRI and PSTI, electrophoresed in 1% agarose gel, then blotted onto nylon membranes. The DNA was probed with a ^{32}P-labelled 2.93-kilobase *sal*I-*Bam*HI fragment of transgene. The blots were exposed onto a BAS-III imaging plate (Fuji Photo Film Co Ltd, Tokyo, Japan), and the transgene copy number was estimated by densitometry (Amersham Pharmacia Biotech AB, Uppsala, Sweden) relative to the ß-actin-ghrelin standard.

Northern blot analysis

Total RNA was denatured with formaldehyde, electrophoresed in 1% agarose gel, then blotted onto a Hybond N^+ membrane (Amersham Pharmacia Biotech AB, Uppsala, Sweden). Mouse ghrelin cDNA obtained by RT-PCR from RNA isolated from mouse stomach was labelled using a digoxigenin labelling kit (Roche Diagnostics KK, Tokyo, Japan). Membranes were hybridised with a digoxigenin labelled cDNA probe for ghrelin mRNA in the stomach, liver, and brain. Total integrated densities of hybridisation signals were determined by densitometry. Data were normalised to G3PDH mRNA abundance.

Determination of body temperature

A lubricated thermocouple was inserted 1.5 cm into the rectum of conscious mice. Rectal temperature was determined using a digital thermometer (Technol Seven Co. Ltd, Yokohama, Japan) in a room maintained at 22°C (SEM 0.5°C).

Oxygen consumption

Oxygen consumption was determined by an O_2/CO_2 metabolism measuring system (Model MK-5000, Muromachikikai, Tokyo, Japan) at 22°C. Chamber volume was 560 ml; airflow to the chamber was 500 ml/min. Samples were taken every three minutes and a standard gas reference was taken every 30 minutes. Mice were kept unrestrained in the chamber without food or water during the light cycle; oxygen consumption was measured for two hours.

Measurement of metabolic parameter and hormone concentrations

Blood was obtained from the orbital sinus under ether anaesthesia. The entire sampling procedure was

done in less than two minutes. Mice were killed by cervical dislocation. Immediately after the mice were killed, the epididymal fat pad mass was removed and weighed. Ghrelin concentrations were measured with RIA using polyclonal rabbit antibodies raised against the amino-terminal (amino acid positions 1 to 11 with O-n-octanoylation at Ser 3) or carboxyl-terminal fragments (amino acid positions 13 to 28) of ghrelin, as previously reported.[1,17,18] Amino-terminal immunoreactivity represents acylated ghrelin alone (N IR), while carboxyl-terminal immunoreactivity corresponds to the sum of both acylated and desacyl ghrelin (C IR). Blood glucose was measured by the glucose oxidase method. Plasma insulin and free fatty acids (FFA) were measured by enzyme immunoassay and enzymatic method (Eiken Chemical Co Ltd, Tokyo, Japan), respectively. Triglycerides and total cholesterol were measured by enzymatic method (Wako Pure Chemical Inds Ltd, Tokyo, Japan). Growth hormone was measured with an immunoradiometric assay kit (Amersham Biosciences, Buckinghamshire, UK).

Statistics

Results are expressed as mean (SEM). Analysis of variance (ANOVA), followed by Bonferroni's *t* test, was used to assess differences among groups. Changes in body weight and nose to anus length were compared using two-way ANOVA. A p value <0.05 was considered to be statistically significant.

RESULTS

▲ TOP
▲ ABSTRACT
▲ METHODS
▪ RESULTS
▼ DISCUSSION
▼ REFERENCES

We investigated whether or not desacyl ghrelin influences feeding behaviour. We first administered desacyl ghrelin into the third cerebral ventricle in non-food deprived mice. Although acylated ghrelin potently increased food intake, desacyl ghrelin showed no increase, but a tendency to decrease food intake compared with ACSF treated control (fig 1⊡). To evaluate the possibility that desacyl ghrelin has an anorexigenic activity, we examined the effects of desacyl ghrelin on food intake in food deprived mice. As shown in fig 2⊡, centrally administered desacyl ghrelin significantly produced inhibitory effects on feeding behaviour. Moreover, we investigated whether intraperitoneally administered desacyl ghrelin has similar effects. Intraperitoneally as well as intracerebroventricularly administered desacyl ghrelin significantly decreased food intake (fig 3⊡). Although a primitive method was used to measure gastric emptying, intracerebroventricular administration of desacyl ghrelin (1 nmol/mouse) reduced the rate of gastric emptying (fig 4⊡). In addition, intraperitoneally administered desacyl ghrelin (3 nmol/mouse) also produced a significant decrease in the gastric emptying rate (fig 5⊡).

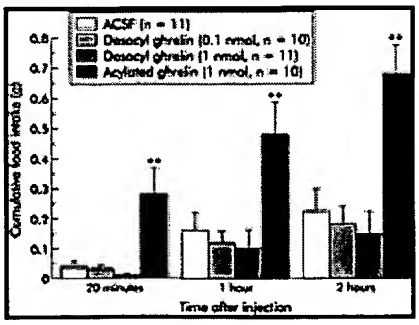


Figure 1 Effects of intracerebroventricularly administered desacyl ghrelin (0.1–1 nmol/mouse) and acylated ghrelin (1 nmol/mouse) on cumulative food intake in non-food deprived mice. Results are expressed as mean (SEM). n indicates the number of mice used. **p<0.01 compared with the artificial cerebrospinal fluid (ACSF) treated control by Bonferroni's *t* test.

View larger version (22K):
[in this window]
[in a new window]

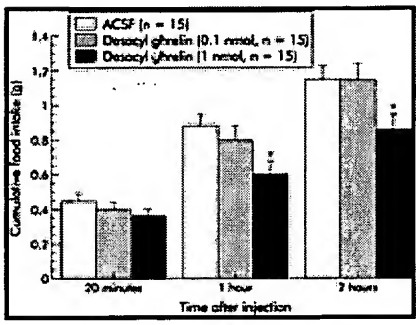


Figure 2 Inhibitory effects of intracerebroventricularly administered desacyl ghrelin (0.1–1 nmol/mouse) on cumulative food intake in 16 hour food deprived mice. Results are expressed as mean (SEM). n indicates the number of mice used. *p<0.05 compared with the artificial cerebrospinal fluid (ACSF) treated control by Bonferroni's *t* test.

View larger version (21K):
[in this window]
[in a new window]

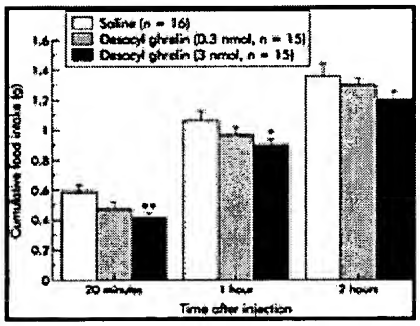


Figure 3 Inhibitory effects of intraperitoneally administered desacyl ghrelin (0.3–3 nmol/mouse) on cumulative food intake in 16 hour food deprived mice. Results are expressed as mean (SEM). n indicates the number of mice used. *p<0.05; **p<0.01 compared with saline treated control by Bonferroni's *t* test.

View larger version (22K):
[in this window]
[in a new window]

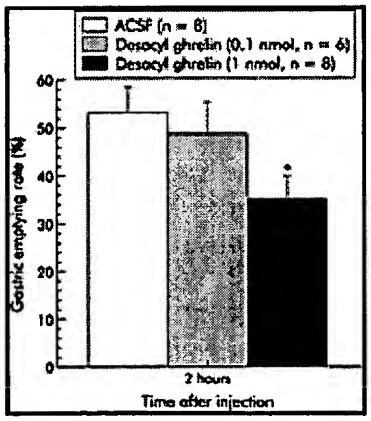
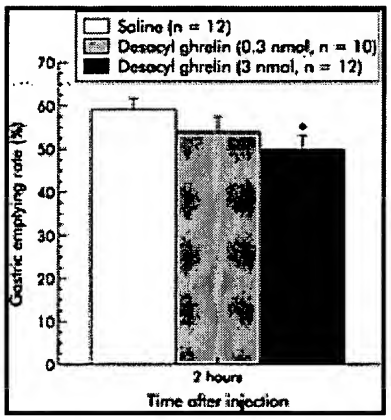


Figure 4 Inhibitory effects of intracerebroventricularly administered desacyl ghrelin (0.1–1 nmol/mouse) on gastric emptying rate two hours after administration. Results are expressed as mean (SEM). n indicates the number of mice used. *p<0.05 compared with the artificial cerebrospinal fluid (ACSF) treated control by Bonferroni's *t* test.

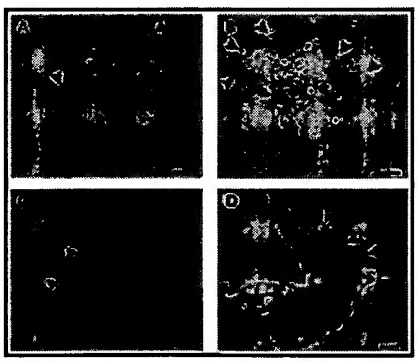
View larger version (21K):
[in this window]
[in a new window]



View larger version (23K):
[in this window]
[in a new window]

Figure 5 Inhibitory effects of intraperitoneally administered desacyl ghrelin (0.3–3 nmol/mouse) on gastric emptying rate two hours after administration. Results are expressed as mean (SEM). n indicates the number of mice used. *$p<0.05$ compared with saline treated control by Bonferroni's *t* test.

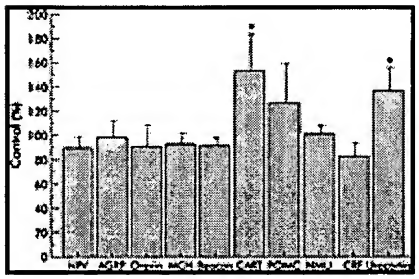
We next examined the effects of peripheral administration of desacyl ghrelin on c-Fos expression in the hypothalamus. Peripheral administration of desacyl ghrelin showed an increase in c-Fos expression in the paraventricular nucleus (PVN) (107 (4.0) *v* 22 (2.0) number/section (control), $p<0.01$) and in the arcuate nucleus (ARC) (40 (8.0) *v* 14.5 (1.5) number/section (control), $p<0.089$) (fig 6). Purple precipitates were distributed mainly in the medial parvocellular part of the PVN and in the ventrolateral part of the ARC. Peripheral administration of desacyl ghrelin showed no significant effects on c-Fos expression in the nucleus tractus solitarius (NTS) of the brainstem (4.0 (2.0) *v* 2.5 (0.5) number/section (control), $p>0.54$). Real-time RT-PCR analysis showed that desacyl ghrelin significantly increased anorexigenic CART and urocortin gene expression (fig 7). Inhibitory effect of desacyl ghrelin administered intraperitoneally (3 nmol/mouse) on feeding was also observed at 90 minutes (1.04 (0.03) *v* 1.19 (0.05) g (control), $p<0.05$) after administration in 12 hour food deprived mice.



View larger version (120K):

Figure 6 Effects of intraperitoneally administered desacyl ghrelin (3 nmol/mouse) on c-Fos expression in the hypothalamus 90 minutes after administration. (A) Paraventricular nucleus (PVN) of saline treated control. (B) PVN of desacyl ghrelin treated mice. (C) Arcuate nucleus (ARC) of saline treated control. (D) ARC of desacyl ghrelin treated mice. c-Fos positive neurons (arrows) were increased dramatically in desacyl ghrelin treated mice. Scale bars = 50 μm.

[in this window]
[in a new window]

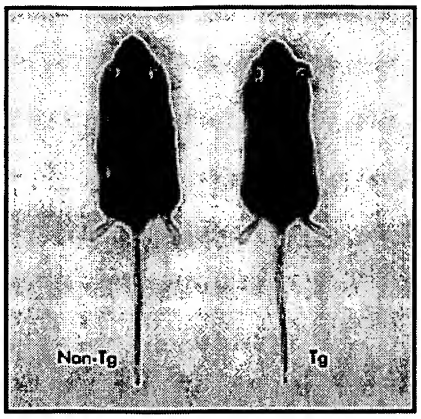


View larger version (23K):
[in this window]
[in a new window]

Figure 7 Effects of intraperitoneally administered desacyl ghrelin (3 nmol/mouse every six hours for 12 hours) on hypothalamic peptides mRNA levels, as assessed by real-time reverse transcription polymerase chain reaction (real-time RT-PCR) in food deprived mice, expressed as a percentage of saline treated control. Results are expressed as mean (SEM) (n = 5 to 6). *p<0.05 compared with the saline treated control by Bonferroni's *t* test. NPY, neuropeptide Y; AGRP, agouti related protein; MCH, melanin concentrating hormone; CART, cocaine and amphetamine regulated transcript; POMC, proopiomelanocortin; NMU, neuromedin U; CRH, corticotropin releasing hormone.

Transgenic mice were generated with overexpression of desacyl ghrelin under control of cytomegalovirus immediate early enhancer-chicken ß-actin promoter (fig 8). Northern blot analysis revealed markedly increased ghrelin mRNA in stomach, brain, and liver tissues of Tg mice with 10 copies of the transgene (fig 9). Plasma desacyl ghrelin concentrations in Tg mice were elevated by approximately 30-fold relative to non-Tg littermates (fig 10). No significant differences in plasma acylated ghrelin concentrations were noted between Tg mice and non-Tg littermates. As shown in table 2, body weights were significantly reduced in Tg mice with a tendency to decrease the linear growth (nose to anus length) ($p<0.097$). Cumulative food intake and fat pad mass weight were also reduced in Tg mice (table 3). Despite the decreased body weight, hypothalamic gene expression of CART (161.7 (31.31) % of control) and urocortin (153.4 (36.47) % of control) showed a tendency to increase in 44 week old Tg mice, although this effect failed to reach statistical significance. Similar findings were observed in body weight (36.70 (1.248) *v* 39.86 (0.755) g (control), $p<0.05$), food intake (28.25 (1.248) *v* 31.07 (0.466) g/week (control), $p<0.05$), fat pad mass (1.346 (0.158) *v* 1.723 (0.088) g (control), $p<0.05$) and linear growth (9.450 (0.242) *v* 9.607 (0.237) cm (control)) of another line (11–1) of 44 week old Tg mice. Tg mice and non-Tg littermates exhibited no significant differences in body temperature. Oxygen consumption showed a tendency to increase in Tg mice (9.481 (0.759) *v* 8.538 (0.374) l/h/kg (control)), although this effect failed to reach statistical significance. The plasma triglycerides level was significantly decreased with the accompanying tendency of the free fatty acids level to decrease moderately. There were no significant differences in plasma growth hormone levels. Gastric emptying rate in 44 week old Tg mice was decreased compared with the non-Tg littermates (fig 11).

Figure 8 Gross appearance of transgenic (Tg) mice overexpressing desacyl ghrelin and non-Tg littermates.



View larger version (126K):
[in this window]
[in a new window]

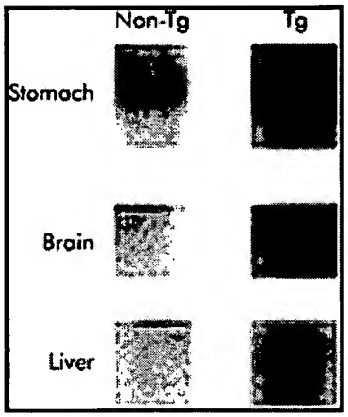


Figure 9 Ghrelin mRNA levels as assessed by Northern blot analysis in Tg mice.

View larger version (49K):
[in this window]
[in a new window]

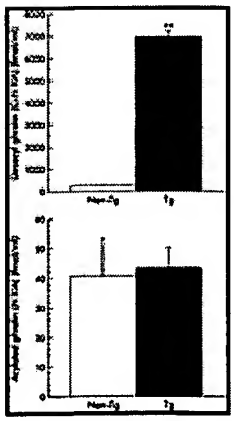


Figure 10 Plasma ghrelin concentrations in Tg mice and non-Tg littermates. Results are expressed as mean (SEM) (n = 3). **p<0.01 compared with the non-Tg littermates by Bonferroni's *t* test. This experiment was repeated in another line (11–1) of Tg mice, with similar results.

View larger version

(11K):
[in this window]
[in a new window]

View this table: [in this window] [in a new window]

Table 2 Changes in body weight and nose to anus length with age in desacyl ghrelin transgenic (Tg) mice and non-Tg littermates

View this table: [in this window] [in a new window]

Table 3 Comparison of food intake, body temperature, epididymal fat mass, metabolic parameters, and growth hormone concentration in 44 week old desacyl ghrelin transgenic (Tg) mice and non-Tg littermates

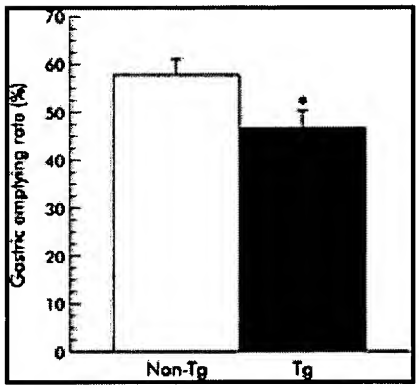


Figure 11 Gastric emptying rate in Tg mice and non-Tg littermates. Results are expressed as mean (SEM) (n = 10). *p<0.05 compared with the non-Tg littermates by Bonferroni's *t* test.

View larger version (11K):
[in this window]
[in a new window]

DISCUSSION

▲ TOP
▲ ABSTRACT
▲ METHODS
▲ RESULTS
▪ DISCUSSION
▼ REFERENCES

In the past few years, much progress has been made in the understanding of how people maintain body weight and how homeostasis is affected.[19–22] In 1999, ghrelin was discovered in the stomach as an appetite stimulatory signal from the periphery with structural resemblance to motilin.[1,4,5,23] Previous studies have shown that acylated ghrelin produces stimulatory effects on food intake via activation of NPY, AGRP, and orexin in the hypothalamus, and on gastric emptying.[4,5,23–25] However, very little is known about the physiological role of desacyl ghrelin. We found that administration of desacyl ghrelin produced inhibitory effects on feeding. Notably, the anorexigenic effect of desacyl ghrelin was inversely proportioned to that of the orexigenic effect of acylated ghrelin. Considerable evidence cumulatively

indicates that rapid gastric emptying is closely related to overeating and obesity, as delayed gastric emptying is to anorexia and cachexia.[26–28] We previously reported that acylated ghrelin stimulates feeding behaviour with its mechanism of action involving the increase of gastric emptying.[23] In the present study, administered desacyl ghrelin decreased the gastric emptying rate. Recently, in vitro desacyl ghrelin has been shown to inhibit cell proliferation in breast cancer cells and cell death in cardiomyocytes and endothelial cells through binding to an unknown receptor that is distinct from GHS-R.[29] In addition, desacyl ghrelin influences contractility of the papillary muscle.[30] These observations indicate that desacyl ghrelin is not devoid of any activities and may have an anorexigenic activity that is contrary to the orexigenic activity of acylated ghrelin.

Neuropeptides in the hypothalamus play a pivotal role in physiological mechanisms regulating food intake and body weight.[19–22] Neuropeptides that stimulate feeding comprise NPY, AGRP, orexin, MCH, and beacon, whereas those that inhibit comprise CART, POMC, NMU, CRH, and urocortin. Previous studies have shown that acylated ghrelin and GHS-R antagonists influence feeding behaviour and gut motility.[4–6,31,32] GHS-R is present in various regions including the hypothalamus and intestine.[1,4,5] In the hypothalamus, GHS-R is located in the ARC, where two orexigenic peptides, NPY and AGRP, are synthesised in the neuron. Up to now, acylated ghrelin has been reported to stimulate feeding behaviour with its mechanism of action involving activation of hypothalamic neurons.[4,5,23–25] In the present study (unpublished data) and in previous reports,[33] c-Fos expression in the ARC and the PVN was increased by administered acylated ghrelin. On the other hand, peripheral administration of desacyl ghrelin also showed an increase in c-Fos expression in the ARC and in the PVN. Gene expression of anorexigenic CART and urocortin in the hypothalamus was increased by administration of desacyl ghrelin. Both CART expressing neurons and urocortin expressing neurons are present in several brain regions that include the hypothalamus.[34,35] Several studies have shown that CART and urocortin are endogenous satiety factors in the central nervous system.[19–22]

Date *et al* recently reported that GHS-R is synthesised in vagal afferent neurons in the nodose ganglion and transported to their afferent terminals in the stomach.[36] It has been demonstrated that desacyl ghrelin has no effects on vagal nerve activities, even though acylated ghrelin acts as an orexigenic signal from periphery to the hypothalamus through the vagal nerve.[23,36] We also found that peripherally administered desacyl ghrelin showed no significant effects on c-Fos expression in the NTS of the brainstem. Banks *et al* recently reported that desacyl ghrelin can cross the blood-brain barrier in the blood to brain direction, although acylated ghrelin enters the brain to a far lesser degree.[37] Taken together, these observations indicate that desacyl ghrelin can act, on hypothalamic sites that are accessible to peripheral hormones, as a feeding suppressor through the endocrine pathway. On the other hand, the receptor for desacyl ghrelin has not been identified. However, our results indicate that desacyl ghrelin decreases food intake with its mechanism of action involving the hypothalamus and stomach. In addition, the inhibitory effect of desacyl ghrelin administered intraperitoneally on feeding was faster than that of desacyl ghrelin administered intracerebroventricularly, suggesting an involvement of peripheral mechanism(s) that are yet to be examined.

To explore the effects of long term overexpression of desacyl ghrelin, we created Tg mice that

overexpress desacyl ghrelin. As expected, the Tg mice gained less weight with reduced fat mass compared with non-Tg littermates. The linear growth showed a tendency to decrease in Tg mice. Consistent with the observation that administration of desacyl ghrelin decreased food intake and gastric emptying, these were reduced in Tg mice. There was no significant difference between Tg mice and non-Tg littermates in body temperature and oxygen consumption. Therefore, this thin phenotype of the Tg mice may be due, at least in part, to a decrease in food intake.

These findings indicate that desacyl ghrelin induces a state of negative energy balance and body weight decrease by inhibiting food intake in an inverse manner to acylated ghrelin. The effect of ghrelin on adipogenesis still remains to be determined. In bone marrow, Thompson *et al* recently reported that acylated ghrelin and desacyl ghrelin stimulate tibial bone marrow adipogenesis via a receptor other than GHS-R.[38] On the other hand, Zhang *et al* reported that ghrelin inhibits adipogenesis in 3T3-L1 cell line by stimulation of preadipocyte cell proliferation via a novel ghrelin receptor subtype.[39] The present study shows that effects of administered desacyl ghrelin on food intake and gastric emptying were contrary to those of acylated ghrelin. Moreover, desacyl ghrelin overexpressing mice showed thin phenotype with decreased food intake and gastric emptying rate. Therefore, the distinction between acylated ghrelin and desacyl ghrelin may be needed to investigate physiological and pathological functions of ghrelin. Previous studies have shown that plasma ghrelin concentration is increased by fasting and decreased by feeding.[4,5,17] Recently, Ariyasu *et al* reported that 70% food restriction decreases the ratio of desacyl ghrelin to acylated ghrelin in food restricted mice compared with ad libitum fed mice.[18]

Thus, the stomach may regulate energy balance via acylated ghrelin and desacyl ghrelin as an endocrine organ. Eating abnormalities are associated with various diseases including obesity, diabetes, anorexia nervosa, and cachexia. Better understanding of the role of ghrelin peptides may provide an entirely new therapeutic approach for treatment of these various diseases, which have become increasingly prevalent throughout the world.

ACKNOWLEDGEMENTS

The authors thank Dr Hiroshi Hosoda and Professor Kenji Kangawa (National Cardiovascular Center Research Institute, Osaka, Japan) for measurement of ghrelin concentration, Toshihiro Kaga and Miwako Katagi (Kobe University, Kobe, Japan) for technical assistance, and Professor Toshifumi Sakai (Saitama University, Saitama, Japan) for discussion.

REFERENCES

▲ TOP
▲ ABSTRACT
▲ METHODS
▲ RESULTS
▲ DISCUSSION
▪ REFERENCES

1. **Kojima M** , Hosoda H, Date Y, *et al.* Ghrelin is a growth-hormone-releasing acylated peptide from stomach. *Nature* 1999;**402**:656–60.[CrossRef] [Medline]

2. **Hosoda H** , Kojima M, Matsuo H, *et al.* Ghrelin and des-acyl ghrelin: two major forms of rat ghrelin peptide in gastrointestinal tissue. *Biochem Biophys Res Commun* 2000;**279**:909–13.[CrossRef][Medline]
3. **Hosoda H** , Kojima M, Mizushima T, *et al.* Structural divergence of human ghrelin. Identification of multiple ghrelin-derived molecules produced by post-translational processing. *J Biol Chem* 2003;**278**:64–70.[Abstract/Free Full Text]
4. **Inui A** . Ghrelin: an orexigenic and somatotrophic signal from the stomach. *Nat Rev Neurosci* 2001;**2**:551–60.[CrossRef][Medline]
5. **Kojima M** , Kangawa K. Ghrelin, an orexigenic signaling molecule from the gastrointestinal tract. *Curr Opin Pharmacol* 2002;**2**:665–8.[CrossRef][Medline]
6. **Fujino K** , Inui A, Asakawa A, *et al.* Ghrelin induces fasted motor activity of the gastrointestinal tract in conscious fed rats. *J Physiol* 2003;**550**:227–40.[Abstract/Free Full Text]
7. **Nagaya N** , Kangawa K. Ghrelin improves left ventricular dysfunction and cardiac cachexia in heart failure. *Curr Opin Pharmacol* 2003;**3**:146–51.[CrossRef][Medline]
8. **Carlini VP**, Monzon ME, Varas MM, *et al.* Ghrelin increases anxiety-like behavior and memory retention in rats. *Biochem Biophys Res Commun* 2002;**299**:739–43.[CrossRef][Medline]
9. **Cummings DE**, Weigle DS, Frayo RS, *et al.* Plasma ghrelin levels after diet-induced weight loss or gastric bypass surgery. *N Engl J Med* 2002;**346**:1623–30.[Abstract/Free Full Text]
10. **Tschop M** , Smiley DL, Heiman ML. Ghrelin induces adiposity in rodents. *Nature* 2000;**407**:908–13.[CrossRef][Medline]
11. **Yoshimoto A** , Mori K, Sugawara A, *et al.* Plasma ghrelin and desacyl ghrelin concentrations in renal failure. *J Am Soc Nephrol* 2002;**13**:2748–52.[Abstract/Free Full Text]
12. **Barrachina MD**, Martinez V, Wang L, *et al.* Synergistic interaction between leptin and cholecystokinin to reduce short-term food intake in lean mice. *Proc Natl Acad Sci U S A* 1997;**94**:10455–60.[Abstract/Free Full Text]
13. **Asakawa A** , Inui A, Yuzuriha H, *et al.* Characterization of the effects of pancreatic polypeptide in the regulation of energy balance. *Gastroenterology* 2003;**124**:1325–36.[CrossRef][Medline]
14. **Roh J** , Chang CL, Bhalla A, *et al.* Intermedin is a calcitonin/calcitonin gene-related peptide family peptide acting through the calcitonin receptor-like receptor/receptor activity-modifying protein receptor complexes. *J Biol Chem* 2004;**279**:7264–74.[Abstract/Free Full Text]
15. **Niwa H** , Yamamura K, Miyazaki J. Efficient selection for high-expression transfectants with a novel eukaryotic vector. *Gene* 1991;**108**:193–9.[CrossRef][Medline]
16. **Southern EM**, Mitchell AR. Chromatography of nucleic acid digests on thin layers of cellulose impregnated with polyethyleneimine. *Biochem J* 1971;**123**:613–17.[Medline]
17. **Ariyasu H** , Takaya K, Tagami T, *et al.* Stomach is a major source of circulating ghrelin, and feeding state determines plasma ghrelin-like immunoreactivity levels in humans. *J Clin Endocrinol Metab* 2001;**86**:4753–8.[Abstract/Free Full Text]
18. **Ariyasu H** , Takaya K, Hosoda H, *et al.* Delayed short-term secretory regulation of ghrelin in obese animals: evidenced by a specific RIA for the active form of ghrelin. *Endocrinology* 2002;**143**:3341–50.[Abstract/Free Full Text]
19. **Inui A** . Transgenic approach to the study of body weight regulation. *Pharmacol Rev* 2000;**52**:35–61.[Abstract/Free Full Text]
20. **Spiegelman BM**, Flier JS. Obesity and the regulation of energy balance. *Cell* 2001;**104**:531–43. [Medline]
21. **Dhillo WS**, Bloom SR. Hypothalamic peptides as drug targets for obesity. *Curr Opin Pharmacol* 2001;**1**:651–5.[CrossRef][Medline]
22. **Marx J** . Cellular warriors at the battle of the bulge. *Science* 2003;**299**:846–9. [Abstract/Free Full Text]
23. **Asakawa A** , Inui A, Kaga T, *et al.* Ghrelin is an appetite-stimulatory signal from stomach with structural resemblance to motilin. *Gastroenterology* 2001;**120**:337–45.[Medline]
24. **Nakazato M** , Murakami N, Date Y, *et al.* A role for ghrelin in the central regulation of feeding.

Nature 2001;**409**:194–8.[CrossRef][Medline]
25. **Toshinai K** , Date Y, Murakami N, *et al.* Ghrelin-induced food intake is mediated via the orexin pathway. *Endocrinology* 2003;**144**:1506–12.[Abstract/Free Full Text]
26. **Duggan JP**, Booth DA. Obesity, overeating, and rapid gastric emptying in rats with ventromedial hypothalamic lesions. *Science* 1986;**231**:609–11.[Medline]
27. **Inui A** . Cancer anorexia-cachexia syndrome: are neuropeptides the key? *Cancer Res* 1999;**59**:4493–501.[Abstract/Free Full Text]
28. **Inui A** . Cancer anorexia-cachexia syndrome: Current issues in research and management. *CA-Cancer J Clin* 2002;**52**:72–91.[Abstract/Free Full Text]
29. **Baldanzi G** , Filigheddu N, Cutrupi S, *et al.* Ghrelin and des-acyl ghrelin inhibit cell death in cardiomyocytes and endothelial cells through ERK1/2 and PI 3-kinase/AKT. *J Cell Biol* 2002;**159**:1029–37.[Abstract/Free Full Text]
30. **Bedendi I** , Alloatti G, Marcantoni A, *et al.* Cardiac effects of ghrelin and its endogenous derivatives des-octanoyl ghrelin and des-Gln14-ghrelin. *Eur J Pharmacol* 2003;**476**:87–95. [CrossRef][Medline]
31. **Asakawa A** , Inui A, Kaga T, *et al.* Antagonism of ghrelin receptor reduces food intake and body weight gain in mice. *Gut* 2003;**52**:947–52.[Abstract/Free Full Text]
32. **Inui A** , Asakawa A, Bowers CY, *et al.* Ghrelin, appetite, and gastric motility: the emerging role of the stomach as an endocrine organ. *FASEB J* 2004;**18**:439–56.[Abstract/Free Full Text]
33. **Lawrence CB**, Snape AC, Baudoin FM, *et al.* Acute central ghrelin and GH secretagogues induce feeding and activate brain appetite centers. *Endocrinology* 2002;**143**:155–62. [Abstract/Free Full Text]
34. **Elias CF**, Lee CE, Kelly JF, *et al.* Characterization of CART neurons in the rat and human hypothalamus. *J Comp Neurol* 2001;**432**:1–19.[CrossRef][Medline]
35. **Skelton KH**, Owens MJ, Nemeroff CB. The neurobiology of urocortin. *Regul Pept* 2000;**93**:85–92.[CrossRef][Medline]
36. **Date Y** , Murakami N, Toshinai K, *et al.* The role of the gastric afferent vagal nerve in ghrelin-induced feeding and growth hormone secretion in rats. *Gastroenterology* 2002;**123**:1120–8. [CrossRef][Medline]
37. **Banks WA**, Tschop M, Robinson SM, *et al.* Extent and direction of ghrelin transport across the blood-brain barrier is determined by its unique primary structure. *J Pharmacol Exp Ther* 2002;**302**:822–7.[Abstract/Free Full Text]
38. **Thompson NM**, Gill DA, Davies R, *et al.* Ghrelin and des-octanoyl ghrelin promote adipogenesis directly in vivo by a mechanism independent of the type 1a growth hormone secretagogue receptor. *Endocrinology* 2004;**145**:234–42.[Abstract/Free Full Text]
39. **Zhang W** , Zhao L, Lin TR, *et al.* Inhibition of adipogenesis by ghrelin. *Mol Biol Cell* 2004;**15**:2484–91.[Abstract/Free Full Text]

This article has been cited by other articles: (Search Google Scholar for Other Citing Articles)

GUT An International Journal of Gastroenterology and Hepatology ▸HOME

T L Peeters
Ghrelin: a new player in the control of gastrointestinal functions
Gut, November 1, 2005; 54(11): 1638 - 1649.
[Full Text] [PDF]